DIVERSITY OF EUKARYOTIC MICROBES IN THE GUT OF ANIMALS AND HUMANS FROM RURAL AND SUBURBAN AREAS

VASANA JINATHAM

DOCTOR OF PHILOSOPHY
IN
BIOLOGICAL SCIENCE

SCHOOL OF SCIENCE
MAE FAH LUANG UNIVERSITY
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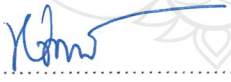

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Vasana Jinatham

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Author	Vasana Jinatham
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ABSTRACT

Eukaryotic microbiome refers to the microscopic eukaryotes that live in the gastrointestinal (GI) tract. The microbial eukaryotes occupying the intestinal tract of humans and animals are fungi and protists. Previously, the majority of protist species in the gut were considered infectious and therefore harmful. Thus, most previous studies focused on the taxonomy and diversity of gut protists found in the gut of people with gastrointestinal symptoms, mainly diarrhea. However, recent works have shown presence of protists in individuals with healthy guts suggesting that these organisms are at least commensal, rather than parasitic. Recent research results have also shown that the GI tract of residents of rural areas contains more diverse and abundant eukaryotic microbes than that of urban residents. Moreover, specific protists have been associated with increased bacterial community richness and evenness and/or abundance of bacteria considered beneficial for gut health. Therefore, knowledge on the overall diversity of gut protists will contribute towards understanding associations with human gut health and disease states. Hence, this study focused on (1) investigating the diversity, prevalence, and distribution of select protists in the gut of animals and humans living in rural and suburban areas of Thailand, and (2) examining the occurrence, diversity, and transmission dynamics of microbial eukaryotes in residents of this study area.

Stool samples were collected from 238 adult Thai nationals from rural and suburban areas living in Chiang Rai (CR), Phayao (PY) and Chachoengsao (CH) provinces. Human volunteers had no history of gastrointestinal diseases and no gastrointestinal symptoms at the time of sampling. Stool samples from 60 animals, namely six pigs, 34 chickens, four buffalo and 16 ectotherms were also collected. We also acquired 55 environmental samples, seven from soil and 48 from water sources. A small amount from each sample was placed in LYSGM media at 37 °C for three days and observed using a compound microscope. All samples were screened for *Blastocystis*, Entamoebidae, Parabasalia, enteromonads and *Balantidium coli* using the small subunit ribosomal RNA (18S rRNA). Phylogenetic analysis was used to assess the diversity of the microbial eukaryotes identified in the samples.

Herein, a new species of *Entamoeba* was isolated from the gut of Asian swamp eel (*Monopterus albus*), an ectotherm living in northern Thailand. Morphological (performed in live and stained cells) and phylogenetic analysis revealed that the new organism is closely related to *Entamoeba invadens*. The new species is established as *Entamoeba chiangraiensis*. These results add to the *Entamoeba* species that have been isolated from fish and provide the first molecular data of its kind.

Following screening of human stool samples, the green alga *Prototheca bovis* was found in four individuals living in a rural area in Thailand. Pure cultures were established for all four samples. The life cycle of the organism was observed and its stages were characterized in detail over a 36-hour period using the Nikon 80i compound microscopy system. Molecular characterization of all four isolates was also performed using the 18S rRNA and cytochrome b (cytb) genes. The life cycle of *P. bovis* resembles that of zoosporic fungi, with up to eight zoospores per mature sporangium. Phylogenetic analysis showed that *Prototheca* was not monophyletic but split into at least two distinct clades. This is the first report of *P. bovis* in human stool samples of individuals with no gastrointestinal symptoms.

To investigate prevalence and transmission dynamics of *Blastocystis*, a rural community in northern Thailand was used as the study area. Various transmission modes such as human-to-human, animal to human and environment to human were examined. Of these, the role of the environment on transmission of *Blastocystis* is mostly unknown. For this study, humans (n=45), animals (n=44) and the environment (n=35) were sampled. *Blastocystis* was present in 73% of human and 100% of animal hosts, while 91% of environmental samples were positive. Overall, ten subtypes were identified: ST1, ST2, ST3, ST4 ST5, ST6, ST7, ST10, ST23, and ST26. Eight of these were detected in humans: ST1, ST2, ST3, ST4, ST5, ST7, ST10, and ST23. The latter two are reported in Thai adults for the first time. It is also the first instance of ST23 in human hosts worldwide. Three subtypes were detected in other animals (ST6, ST7, and ST23), while seven (ST1, ST3, ST6, ST7, ST10, ST23, and ST26) were found in the environment. Five subtypes of *Blastocystis* were shared between humans and the environment (ST1, ST3, ST7, ST10, ST23), while three (ST7, ST10, ST23) overlapped between the environment and animal hosts. No subtypes were shared between humans living in the same residence suggesting that human-to-human transmission did not occur in the examined households. The results indicate that in the community under study, the major transmission route of *Blastocystis* to humans is the environment. This study identified and proposed soil as a novel transmission route of this eukaryote. Moreover it shows that using the One Health perspective, whereby humans, animals and the environment are considered collectively, can help understand transmission patterns of organisms and therefore contribute in developing effective control strategies against pathogens.

Future studies should focus on investigating larger communities and other areas in Thailand under the One Health umbrella. Moreover, microbial eukaryotes carriage patterns in different communities should be investigated along with their metabolome profiles to better understand their roles in gut health and disease.

Keyword: Microbial Eukaryotes, Transmission Mode, Thailand, 18S rRNA

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CHAPTER 1

INTRODUCTION

Non-communicable diseases (NCDs) are increasing worldwide and over 70% of all deaths globally are from NCDs (World Health Organization [WHO], 2018). Overweight and obesity are major risk factors for many NCDs, including diabetes, cardiovascular diseases and cancer. In 2013, prevalence of overweight and obesity in Thai adults was the second highest amongst the nations of the ASEAN, equivalent to 32.2% of all population. Factors of obesity include behavior and genetics and community environment. Recent research studies suggest that composition of gut microbiota may also be a key factor of overweight and obesity problem. Gut microbiota comprise microbial prokaryotes and eukaryotes and play an essential role in health and disease. Recently, research has shown that gut microbiota have several effects on the human host, the best known example being fermentation of dietary fiber. In general healthy and balanced microbiota are diverse, while unhealthy microbiota are not as diverse and their composition is not balanced. Geographic influences, culture, society, and economics affect dietary intake leading to different diversity and composition of gut microbes (Drewnowski & Kawachi, 2015). Most previous studies were focused on taxonomic surveys of diversity of gut microbial prokaryotes from industrialized, urban populations, while rural populations have not been observed as much. The few studies on rural populations have shown that they have very unique composition of gut microbiota. Thus these populations are very important because they are potential sources of novel microbiota species. Similarly, studies focusing on microbial gut eukaryotes are lacking as they were only reported when they had negative effects on the host. However, not all gut eukaryotes are parasitic, but many are commensal and may have important role in health and disease. Moreover, most research of functions and importance of microbial eukaryotes in

animal and human are unclear. However, gut eukaryotes are very strongly associated with specific bacterial microbiota profiles. This implies that microbial eukaryotes can be used to alter composition of bacterial gut microbiota for the benefit of the host. To date, there have been no studies in Thailand examining eukaryotic diversity of gut microbiota in rural and suburban areas using molecular analysis

To bridge these gaps, this study aims to investigate the diversity, prevalence, and distribution of the eukaryotic microbiome of animals and humans living in rural and suburban areas of Thailand. Samples will be collected and a combination of light microscopy, cell culturing and molecular techniques will be used to identify microbial eukaryotes. The project will provide significant knowledge on the eukaryotes present in the gut of Thai adults and their animals and pave the way for future interventions.

Reference

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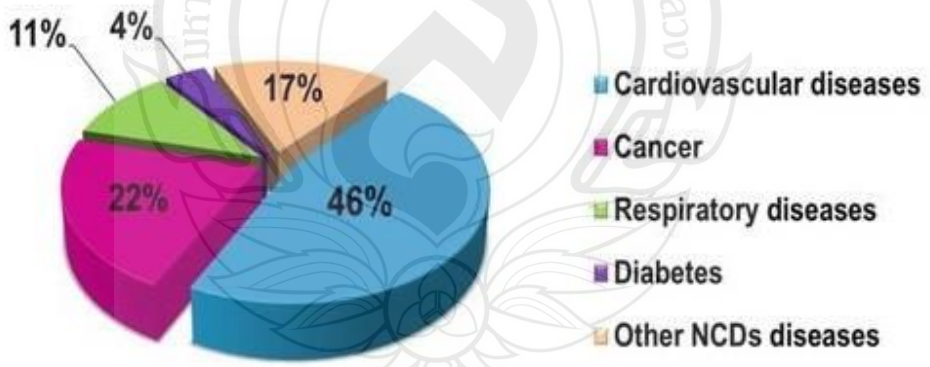
World Health Organization (WHO). (2018). *Noncommunicable diseases*. https://www.who.int/news-room/fact-sheets/detail/noncommunicable-diseases

CHAPTER 2

LITERATURE REVIEWS

2.1 Non-communicable Diseases

In recent decades, non-communicable diseases (NCDs), also known as non-infectious diseases, have become a major problem worldwide. The NCDs are diseases that are not passed from person to person. The top five NCDs include cardiovascular diseases, cancers, chronic respiratory diseases, diabetes and gastrointestinal diseases (WHO, 2018). In 2014, the world health organization (WHO) reported that more than 70 percent of people die from a NCD between the ages of 30 and 69 years. Of these, cardiovascular diseases were responsible for 46% of these deaths, cancers for 22%, chronic respiratory diseases for 11%, and diabetes for 4% (Figure 2.1)



Note Cardiovascular diseases are the top NCD cause of death

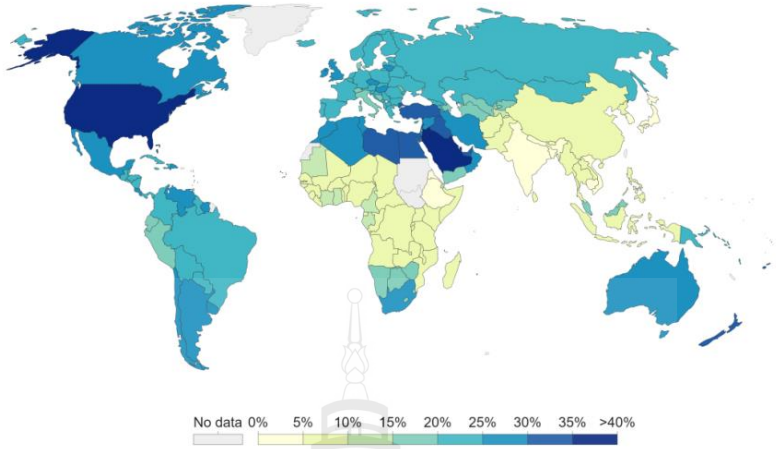
Source Mastrangelo and Barbas (2017)

Figure 2.1 Percentage of deaths from non-communicable diseases (NCDs) in 2012

Risk factors of NCDs include modifiable behaviors, such as tobacco use, physical inactivity, unhealthy diet and the harmful use of alcohol. Metabolic factors, such as raised blood pressure, overweight/obesity, hyperglycemia and hyperlipidemia also constitute risk (WHO, 2018). Amongst these, overweight/obesity is one of the main causes of NCDs worldwide, including Thailand (Webber et al., 2012).

2.2 Obesity

Obesity is defined as abnormal or excessive fat accumulation that leads to health problems including type 2 diabetes, coronary heart disease (CAD) and gastrointestinal cancers. The number of overweight/obese in the world has more than doubled since 1980 and it has increasing trend (WHO, 2016). In 2016, the WHO reported that more than 1.9 billion adults were overweight, while over 650 million were obese worldwide. The highest percentage of obesity rate in the world was found in United States (30.6%), Mexico (24.2%), United Kingdom (23%), Slovakia (22.4%), Greece (21.7%), Australia (21.9%), New Zealand (20.9%), Hungary (18.8%), Luxembourg (18.4%) and Czech Republic (14.8%), whereas in Thailand obesity prevalence was 10% and was shown in figure 2.2 (Ritchie & Roser, 2016)



Note Individuals with body- mass index (BMI) greater than 30 kilograms per meter squared were considered obese

Source Ritchie and Roser, 2016

Figure 2.2 The prevalence of obesity in male and female adults

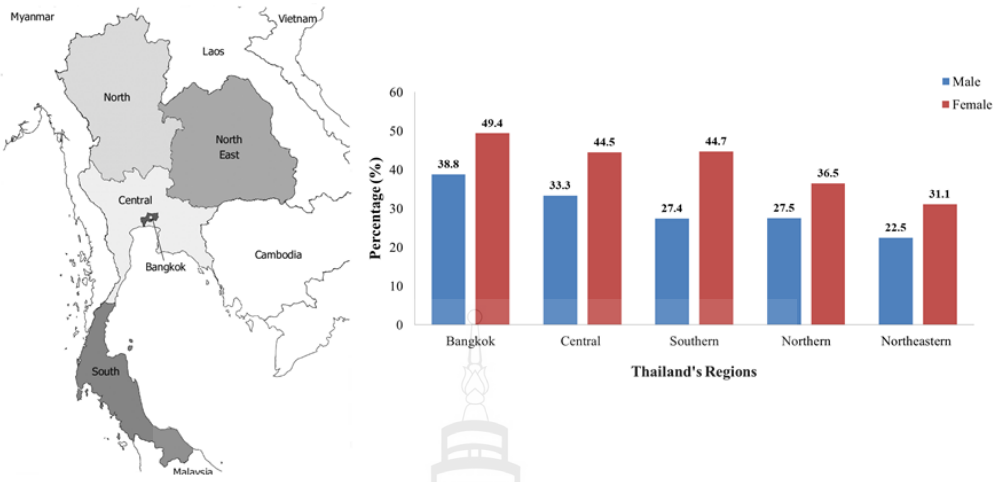
In 2013, the prevalence of overweight and obesity combined in the Thai adult population increased and Thailand became the second most obese country in the ASEAN region at 32.2% which was shown in figure 2.3 (Bickerstaff, 2013; Nation in Southeast Asia, 2014).

Note Thai people were reported as the second rank in the top five Asia-Pacific nations

Source Bickerstaff (2013)

Figure 2.3 Percent prevalence of overweight and obesity in adults of both sexes with BMI >25kg/m^2

Population of people living in Bangkok had the highest prevalence of obesity for both males (38.8%) and females (49.4%), whereas prevalence was lower in the central (33.3% for males and 44.5% for females), southern (27.4% and 44.7%), northern (27.5% and 36.3%) and north-eastern regions (22.5% and 39.1%) (Figure 2.4)



Note The highest percentage of overweight and obesity adults lives in Bangkok

Source Teerawattananon and Luz (2017)

Figure 2.4 Percentage of overweight and obesity in Thai adults of both sexes

Factors such as growing economy, technology, and food availability contribute to the obesity problem (Brehm & D'Alessio, 2014; Siddarth, 2013). Those factors lead to change of life style and eating behavior. For example, people have greater exposure to processed foods and soft drinks, readily available at convenience stores, so they consume more of these products. Moreover, there is increasingly reduction in the amount of physical activity undertaken (Montgomerie et al., 2014; Pietiläinen et al., 2008; Stankov et al., 2012). However, most studies suggest that food behavior and physical activity environments are the main causes of energy imbalance, which in turn leads to obesity (Bahreynian et al., 2013; DeWeese et al., 2018; Drewnowski et al., 2016; Goularte et al., 2012; Kolodinsky & Goldstein, 2011). For example, Siddarth (2013) showed that eating habits and physical activity are associated with obesity, while Chan et al. (2017) reported that low level of physical activity relate with overweight/obesity in Malaysian adults. Energy imbalance is the relationship between calorie intake and energy expenditure and is thought as one of the factors determining a person's weight (Butte et al., 2007; Hill et al., 2012; Romieu et al., 2017).

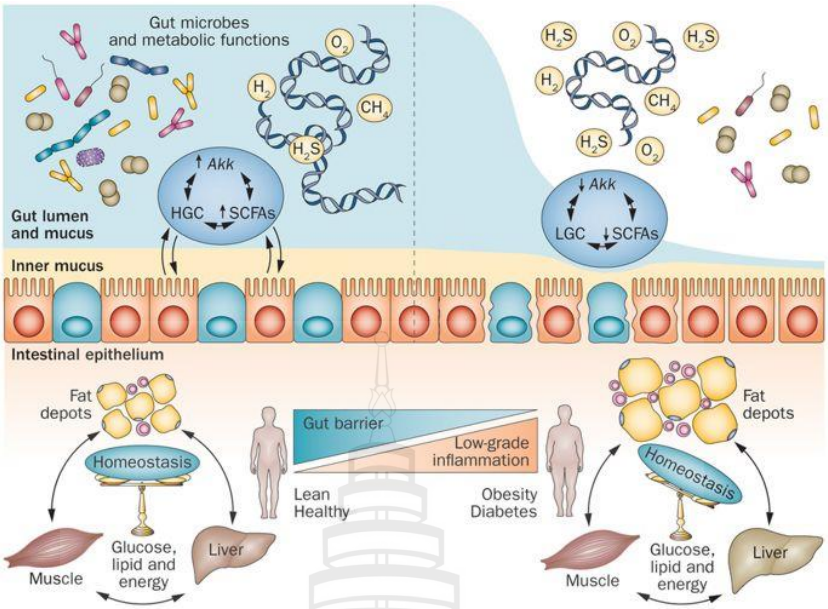
Most of the energy is received from foods such as carbohydrate, protein and fat (Bowman & Vinyard, 2004; Drewnowski & Darmon, 2005; Phillips et al., 2004). For example, healthy dietary pattern can reduce overweight/obesity risk, whereas unhealthy dietary pattern has an effect on increasing overweight/obesity (Flores et al., 2010; Giskes et al., 2010; Gittelsohn et al., 1998; Mu et al., 2017; Popkin, 2001). In recent years, another factor was discovered to play a critical role in energy imbalance. That factor is the gut microbiota, which derive energy from carbohydrate fermentation yielding short chain fatty acids (SCFA) and are important on energy homeostasis and metabolism (den Besten et al., 2013; Tan et al., 2014).

2.3 Gut Microbiota

Gut microbiota represents an ensemble of microorganisms living in the gastrointestinal (GI) tract. Human gut microbiota consists of bacteria, archaea, viruses and eukaryotic microbes all of which play an essential role in digestion of complex carbohydrate and host–derived glycans in the human intestine (Flint et al., 2012). Gut microbes have many roles: they break down xenobiotics in both beneficial and potentially harmful ways (Clarke et al., 2019). Some microbes, such as probiotic bacteria, can synthesize de novo and supply vitamins to human body, such as, vitamin K, as well as most of the water-soluble B vitamins,(cobalamin, folates, pyridoxine, riboflavin, and thiamine) (Hill, 1997; LeBlanc et al., 2013; Degnan et al., 2014; Magnúsdóttir et al., 2015). Gut microbiota regulates innate and adaptive immune homeostasis and can affect both positively and negatively to systemic autoimmune diseases (Wu & Wu, 2012; Shi et al., 2017).

Finally, gut microbes strengthen gut integrity, shape the intestinal epithelium, harvest energy, protect against pathogens and regulate host immunity (Morrison & Preston, 2016). However, some gut microbiota taxa have a close link with human diseases such as infectious diseases, liver diseases, gastrointestinal cancers, metabolic diseases, respiratory diseases, mental or psychological diseases and autoimmune diseases (Wang et al., 2017).

Research has shown that gut microbiota have several effects on the human host. These effects can be both positive and negative (Cani, 2018; Kho & Lal, 2018; Valdes et al., 2018; Zhang et al., 2015). For example, bacteria produce SCFAs, which are source of energy of colon cells and reduce leaky gut (Figure 2.5) (Chambers et al., 2018; den Besten et al., 2013; Kuwahara, 2014). Moreover, some bacteria produce mucin forming a layer of mucus, which has protective function for the gut. The inner mucus layer can be disrupted in disease, allowing access of organisms to the underlying epithelium (Corfield, 2018; Derrien et al., 2010; Sicard et al., 2017). On the other hand, a negative effect is that some gut bacteria can increase intestinal permeability for bacterial lipopolysaccharides (LPS) in response to the consumption of high-fat diets. In turn this results in an elevated systemic LPS level and low-grade inflammation (Blaut, 2015; Cani et al., 2012; DiBaise et al., 2008; Heiss & Olofsson, 2018; Nam et al., 2008; Rowland et al., 2018; Yang & Kweon, 2016).



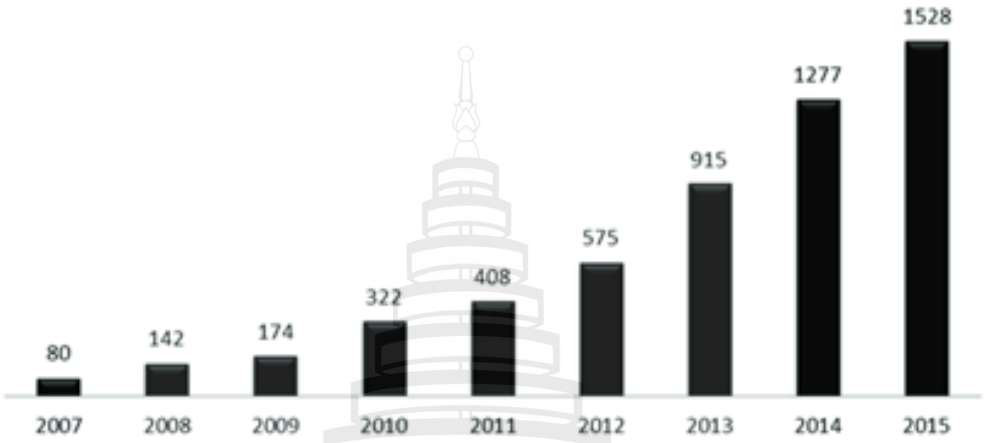
Note The left part of the figure shows that in the healthy gut, the composition of gut microbiome is associated with a higher mucus layer thickness and high SCFAs production, which contribute to reduced food intake and improved glucose metabolism. The right part of the figure shows less diverse composition of gut microbiota related with low mucus layer thickness and low SCFAs, were the key factors triggering the onset of low-grade inflammation and insulin resistance

Source Cani (2014)

Figure 2.5 Effect of gut microbiota and metabolic functions on human health

Many studies have shown that balanced microbiota is characterized by diverse communities of live microorganisms in healthy humans and are linked to high gut epithelial integrity. Most of these diverse bacteria are members of the divisions Bacteroidetes and Firmicutes. Loss of gut epithelial integrity leading to gastrointestinal problems, such as abdominal bloating, excessive gas and cramps, and food sensitivities is associated with unbalanced and low diversity of gut microbiota (Clemente et al., 2012; Wang et al., 2018).

In recent years, the number of studies on composition and population of human gut microbiota has increased globally (Figure 2.6). The number of publications has increased exponentially over the last decade and is expected to have increasing trend in the future.



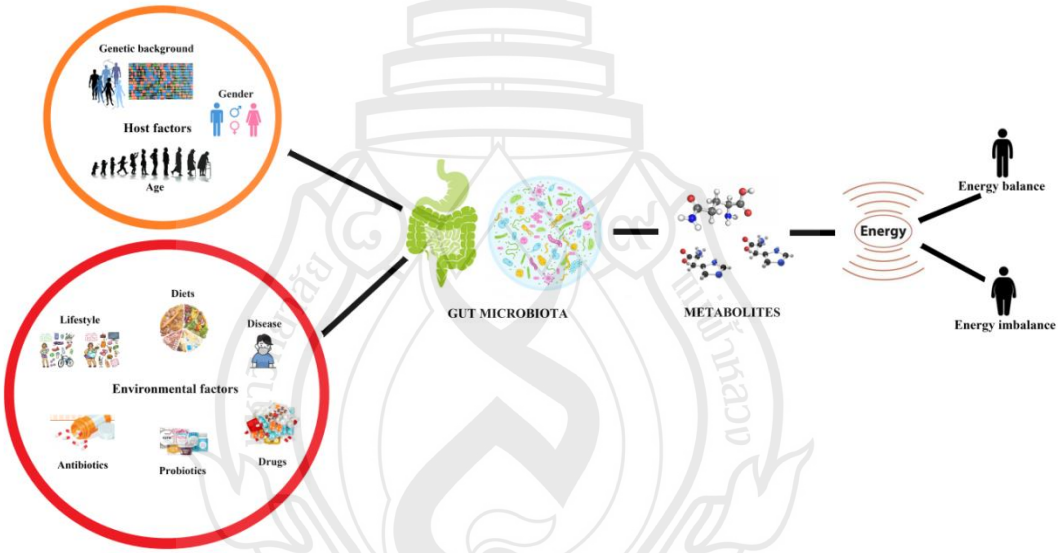
Source Eid et al., 2017

Figure 2.6 Number of publications focusing on human gut microbiota in Pubmed database in the last decade, per year

2.4 Composition of Gut Microbiota

Most previous studies on gut microbiota of Europeans, Americans, Koreans, Africans, Danish, Chinese and populations of other regions have found differences in the composition of gut communities (Deschasaux et al., 2018; Gaulke & Sharpton, 2018; Gupta et al., 2017; Suzuki & Worobey, 2014). These differences are related with multiple factors (Figure 2.7) including host factors (genetic background and gender) and environmental factors (diet, lifestyle, hygiene, and use of antibiotics and probiotics) (Lagier et al., 2012; Kumar et al., 2016; Xu et al., 2017).

Studies on the genetic background of 416 pairs of twins showed that host genetics may be involved in the composition of gut microbiota and lead to imbalance of host metabolism (Dąbrowska & Witkiewicz, 2016; Goodrich et al., 2014; Hall et al., 2017), whereas gender is an indirect factor in shaping gut diversity. Age is also a factor since stages of age relate with the gut microbial ecosystem. For example, in adulthood, diversity of gut microbial composition is higher than other stages of life (Maffei et al., 2017; Mariat et al., 2009; Odamaki et al., 2016; Yatsunenko et al., 2012). Nevertheless, most studies suggest that diversity of gut microbiota depends on diet (Bäckhed et al., 2004; Claesson et al., 2012; De Filippo et al., 2010; den Besten et al., 2013; Gentile & Weir, 2018; Shen, 2017; Wan et al., 2019).



Source Graf et al. (2015) and Gupta et al. (2017)

Figure 2.7 Multiple factors are effect to diversity of gut microbiota lead to human health, including host factors and environmental factors

2.5 Gut Microbiota and Diet

Diet and interplay with gut microbiota are the primary drivers of the long co-evolutionary history between humans and gut microbes (Moschen et al., 2012). The gut microbiota-modulating effects of diet are well known. Dietary patterns are associated with specific gut microbiome composition. For example, vegetarian diets are reported to increase abundance of SCFAs bacteria, such as *Clostridium* cluster XIVa (Kabeerdoss et al., 2012) while Matijašić et al. (2014) reported increased proportions of *Bacteroides*/*Prevotella* group, *Bacteroides thetaiotaomicron, Clostridium clostridioforme,* and *Faecalibacterium prausnitzii* in vegetarians. Similarly, studies on dietary fiber found that resistant starch (RS), inulin, fructo-oligosaccharides and galacto-oligasaccharides, polydextrose also promote growth of SCFA-producing bacterial group and probiotic bacteria (Benus et al., 2010; Costabile et al., 2010; Hooda et al., 2012; Lecerf et al., 2012; Linetzky et al., 2016; Vulevic et al., 2013). In addition, Graf et al. (2015) showed that western diet influences the diversity of microbiota composition more than traditional diets especially the enterotypes bacterial groups. Furthermore, specific foods, such as whole grain products, fruits and nuts were also studied using whole grain products interventions. The study showed that the intervention led to an increased microbial diversity, as well as a rise in the proportion of Firmicutes and a reduction of the Bacteroidetes phylum (Carvalho-Wells et al., 2010; Costabile et al., 2008; Martínez et al., 2013), whereas fruits and nuts were associated with probiotic bacteria, such as, *Bifidobacterium* spp. and *Lactobacillus acidophilus* (Queipo-Ortuño et al., 2012; Vendrame et al., 2011)

Diet depends on the nature of the geography of each particular region and also, culture, society, and economics (Drewnowski & Kawachi, 2015). Taking geography into consideration, dietary intake differs between rural and urban areas. People living in rural communities consume local and traditional foods, whereas urban individuals eat fast-food and sugary drinks (Downs et al., 2012). Some previous research suggests that these differences in dietary intake have an effect on gut microbiota composition (Senghor et al., 2018). This information was supported in the studies of Ayeni et al. (2018) and Ruggles et al. (2018), who examined gut microbiota in rural and urban area

They found that people in urban areas have lower gut microbiota diversity than those with more traditional lifestyles from rural areas. Also, in a different study, composition of gut microbiota in healthy Indians residing in rural and urban areas was different due to dietary intake of the individuals (Das et al., 2018). Martínez et al. (2015) examined the effect of westernization on structure and composition of the human gut microbiota in adults from Papua New Guinea (PNG) and United States (US) and found that modern lifestyle factors affected bacterial population. Specifically, people living in the US had lower dispersal gut microbiota and higher variable selection, while those living in PNG had the opposite effect. Also, US volunteers had lower α-diversity and higher β-diversity, whereas higher α-diversity and lower β-diversity were found in the PNG samples. Moreover, research on gut microbiota of children living in rural and urban regions showed that dietary habits and westernized lifestyle play a role in shaping gut microbiota (De Filippo et al., 2017). For example, the ratio of Bacteroidetes to Firmicutes bacteria was high in rural populations, whereas in urban residents the ratio was much lower. Furthermore, the amount of SCFAs was lower in the urban samples. Studies of distribution of gut microbiota of school children living in rural and urban areas in Asian countries showed that eco-geographical factors and different food consumption habits shape gut microbial communities (La-Ongkham et al., 2015; Nakayama et al., 2015). Most studies suggest that gut microbiota in people from rural areas had a higher level of certain bacterial species that are important for digesting fiber, whereas urban populations were dominated with bacteria that perform fat and protein digestion (De Filippo et al., 2010; De Filippo et al., 2017; Martínez et al., 2015; Rampelli et al., 2015).

Most of the current knowledge on microbiota comes from studies that have focused on communities of prokaryotic microbes (mostly bacteria) from people while eukaryotic microbes are extremely limite

2.6 Microbial Eukaryotes in the Gut

Microbial eukaryotes are mostly unicellular organisms whose cells have a nucleus enclosed within membranes and are more complex than prokaryotes. Microbial eukaryotes present in the gut of animals and humans are protists and fungi (Laforest-Lapointe & Arrieta, 2018). The roles of these eukaryotes are largely unknown. Most previous research studies on eukaryotes focused on using the microscopy in patients with symptoms, whereas individuals with no symptoms were not studied. These studies reported that principally microbial eukaryotes had negative impact on human health (Parfrey et al., 2011). Some examples are microsporidia (*Encephalitozoon cuniculi*) (Stentiford et al., 2016), *Entamoeba histolytica* (Moonah et al., 2013), *Toxoplasma gondii* (Molloy et al., 2013), *Giardia* spp. and *Cryptosporidium* spp. (Kotloff et al., 2013). However, many studies do not agree that microbial eukaryotes are harmful. Recent studies suggest that many microbial eukaryotes, including stramenopiles (*Blastocystis* spp.), diplomonads (*Enteromonas* spp.), amoebozoa (*Entamoeba dispar, Entamoeba coli*) and parabasalids (*Pentatrichomonas hominis, Dientamoeba fragilis*) could be common and nonpathogenic to human health (Lukes et al., 2015; Scanlan et al., 2014). For example, Scanlan et al. (2014), Lhotská et al. (2020) and Yowang et al. (2019) showed that the microbial eukaryote *Blastocystis* is a common and diverse member in the gut of people with no gastrointestinal symptoms in Ireland, Thailand and Czech republic, respectively.

Diversity of microbial eukaryotes in the gut is probably underestimated. The evidence suggests that reporting microbial eukaryotes using microscopy alone is not enough to capture their true diversity. For example, *Entamoeba histolytica* is pathogenic, while *Entamoeba dispar* and *Entamoeba moshkovskii* are non-pathogenic (Ghenghesh et al., 2016). These three look almost identical when using microscopy. Therefore studies of microbial eukaryotes should combine techniques such as microscopy, molecular technique and metabolic analysis. Diversity of fungi in the gut comprises major phyla, such as Ascomycota, Basidiomycota, Zygomycota and Microsporidia (Hugon et al., 2017).

Most gut fungi are yeasts including species of *Saccharomyces*, *Malassezia*, and *Candida* and are detected frequently in human fecal samples (Nash et al., 2017).

Communities of microbial eukaryotes residing in the human gut have low-level species diversity and abundance, but higher inter-individual variability than prokaryotes (Nam et al., 2008; Nash et al., 2017; Ott et al., 2008; Scanlan & Marchesi, 2008). Microbial eukaryotes are highly adapted in host-associated environments and are either mutualistic or commensal (Parfrey et al., 2011).

Communities of microbial eukaryotes in animals are much more diverse than those of humans. Many studies have focused on parasites. For example *Toxocara canis* and T. *cati* were found in 33.9% of dogs and 31.8% of cats (Villeneuve et al., 2015). *Strongyloides stercoralis, Giardia duodenalis* and *Blastocystis* sp. were found in Australian domestic dogs (Gillespie & Bradbury, 2017). Microbial eukaryotes are also found in healthy animals and they may play a role in their GI health (Betts et al., 2018). In this recent study, *Blastocystis* was found in 118 samples from 27 vertebrate species in a South East England zoo. Importantly, animals harboring *Blastocystis* were asymptomatic. Microbial eukaryotes are also present in gut of ruminant animals and also insects.

The role of microbial eukaryotes in the gut is unclear, but likely to have some benefits. Recent studies showed that *Tritrichomonas musculis* activates the mouse host epithelial inflammasome to induce IL-18 release (Chudnovskiy et al., 2016) and type 2 immune responses (Howitt et al., 2016). However this also was related with altered mucosal T cell homeostasis and colitis susceptibility (Escalante et al., 2016). In ruminants, ciliated protozoa are associated with rumen fiber degradation in cattle, sheep, and goats. Moreover, ciliated protozoa were related to a 30% increase of microbial protein and a 10% reduction of methane production (Newbold et al., 2015). Similarly, the gut of termites and cockroaches is full of parabasalid protists, where the family *Cryptocercidae* plays role in xylose digestion (Berlanga & Guerrero 2016).

Recent studies have found that the fungi and protists residing in the gut have interactions with the bacterial microbiomes of the host that both promote and inhibit activity of community microbes (Laforest-Lapointe & Arrieta, 2018; Sam et al., 2017; Scanlan & Marchesi, 2008).

For example, study of probiotic therapy in pouchitis relate with bacterial and fungal microbiota, found that bacterial diversity was increased, while fungal diversity was reduced (Kuehbacher et al., 2006)

2.7 Association of Microbial Eukaryotes and Bacterial Diversity

In recent years, several studies suggest that single-celled eukaryotes, such as *Blastocystis*, *Dientamoeba* and *Entamoeba* are capable of long-term colonization of the human gut. These are commonly found in individuals with a healthy gut and have been proposed as indicators of intestinal microbiota structure (Stensvold & van der Giezen, 2018). An example case is *Blastocystis*, a common microscopic organism that inhabits the gut of animals and humans. It has 28 subtypes (ST). ST1, ST2, ST3 and ST4 are commonly isolated subtypes in humans, whereas ST5-ST9 are found only rarely. In addition, ST6 and ST7 are from birds and are more frequently found in Asia and the Middle East (Scanlan et al., 2015; Stensvold et al., 2007; Tan, 2008). Presence of *Blastocystis* in healthy adults was strongly associated with increase in bacterial alpha diversity and broad changes in beta diversity. It was hypothesized that *Blastocystis* helped lower competition for nutrients and space of strong bacterial competitors that dominated the community thus leading to an increase in bacterial richness and community evenness (Nash et al., 2017; Nieves-Ramírez et al., 2018; Laforest-Lapointe & Arrieta, 2018). Andersen et al. (2015) showed that *Blastocystis* was less prevalent in *Bacteroides* enterotyped samples and clostridial cluster XIV, but was found at high levels in people with *Prevotella* or *Ruminococcus* enterotyped (Andersen & Stensvold, 2016; Forsell et al., 2017). *Blastocystis* subtypes 3 and 4 were inversely correlated with presence of *Akkermansia*, a beneficial bacterium living in the mucus of the gut. Moreover, colonization with *Blastocystis* related with higher bacterial richness and lower BMI (O'Brien Andersen et al., 2016). Consistent with this, Beghini et al. (2017) found that *Blastocystis* was prevalent in gut microbiome of healthy individuals, had inverse association with BMI and was correlated with *Methanobrevibacter smithii*.

Nourrisson et al. (2014) suggested that *Blastocystis* might be linked to microbiota imbalance and decrease of *Faecalibacterium prausnitzii* and *Bifidobacterium* sp. in males with IBS type C.

The effect of *Entamoeba* on bacterial communities of healthy and unhealthy individuals has also been studied. Most species of *Entamoeba* that colonize the gut of humans and other animals are non-pathogenic. For example, *E. coli* and *E. dispar* have been linked to healthy living and have been associated with significantly higher *Faecalibacterium prausnitzii-Escherichia coli* ratio. Diversity of *Entamoeba* spp. was strongly associated with increased diversity and various shifts in composition of the gut bacterial microbiota (Chabé et al., 2017).

An exception to this is *Entamoeba histolytica*, which is the cause of amoebic bloody diarrhea or amoebiasis as it can penetrate the mucosa. Studies on the contribution of intestinal protists to infectious disease have shown that *E. histolytica* significantly promote the composition of intestinal microbiome and especially the abundance of *Prevotella copri* and *Prevotella stercorea* belonging to *Prevotellaceae* group (Burgess et al., 2017; Morton et al., 2015). *Entamoeba histolytica* can survive oxidative stress and establish itself in the intestinal mucosa with the help of bacteria from the family *Entereobacteriaceae*, while causing changes in genes involved in glycolysis and proteasome activity (Varet et al., 2018). Studies of children with diarrhea found that *E. histolytica* was associated with increasing levels of *Prevotella copri*, *Eubacterium* and *Bifidobacterium* spp. and a decrease in *Clostridium*, *Bacteroides*, *Lactobacillus* and *Campylobacter* (Gilchrist et al., 2016; Verma et al., 2012).

Giardia duodenalis, a pathogenic protist, has been associated with abundance of harmful bacterial species, such as *Escherichia coli* (Iebba et al., 2016). Infection with *Giardia* is linked with increasing distribution of facultative and strictly aerobic bacteria, which indicates an unbalanced microbiome (Barash et al., 2017)

Diversity of gut fungi is also significantly lower in comparison to bacterial diversity and specific fungal species are related to bacterial pathogens. *Candida* species, such as *C. albicans* are major human fungal pathogens that cause both mucosal and deep tissue infections. Many studies suggest that the interactions between bacterial and fungal community could play an important role in the

development of invasive candidiasis (Kapitan et al., 2018; Sam et al., 2017). *Candida albicans* is the most abundant and significant species associated with disease and colonization can lead to infection and invasion of host tissues such as mucosal sites (Huffnagle & Noverr, 2013) Moreover, some studies found that prevalence of *Pseudomonas aeruginosa* can inhibit the filamentation of *C. albicans* virulence whereas *C. albicans* has the ability to inhibit the swarming motility of *P. aeruginosa*. In the healthy gut growth of *C. albicans* is inhibited by SCFA bacteria (Laforest-Lapointe & Arrieta, 2018).

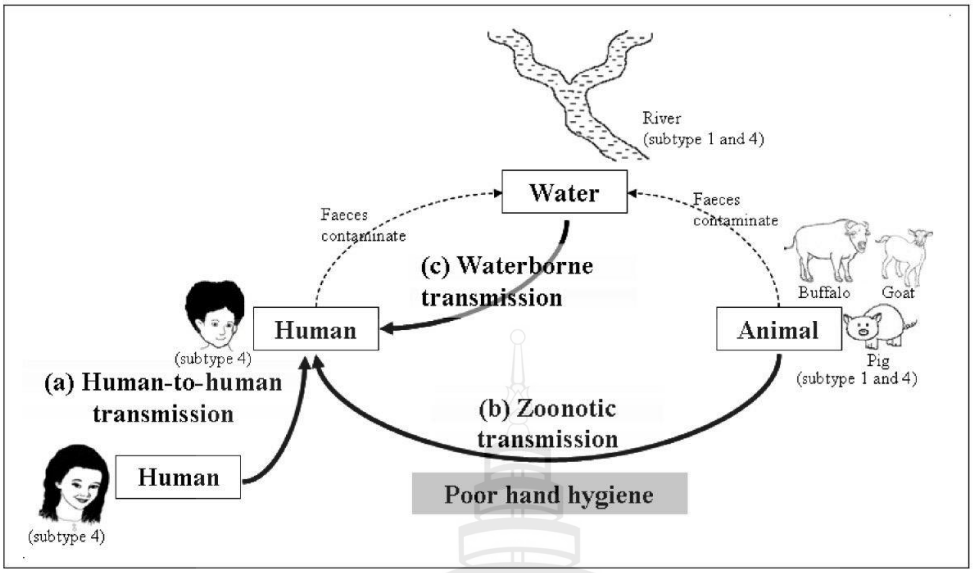
Most of the current knowledge on microbiota comes from interactions between diet factors and gut microbiota. However, knowledge of transmission remains limited

2.8 Gut Microbiota and Transmission Mode

Gut microbes have various modes of transmission. The fecal oral route is significant in the transmission of microbial taxa to humans via poor personal hygiene, human and animal contact, and environmental conditions like contamination of soil and water sources (Tan, 2008; Eroglu & Koltas, 2010; Ii Lee et al., 2012; Alfellani et al., 2013b; Ahmed et al., 2018; Maloney et al., 2019) Epidemiological research carried out in different countries has shown that animal gut microbiota is one of the important factors relating with diversity of human gut microbiota (Figure 2.8). Some taxa from animal microbiota can transfer to humans via diet, water, vegetables, raw or undercooked meat, raw milk or dairy products, environment, and direct contact. These taxa enhance and maintain microbial diversity in the human gut (Browne et al., 2016; Harmsen & de Goffau, 2016; Ley et al., 2006). Previous studies have focused only on gastrointestinal infectious diseases caused by bacteria, viruses, and parasites transmitted from animals to humans lead to humans. These infections are most prevalent in developing countries and, cause human public-health problems. For example, a study of *Entamoeba histolytica* in humans and animals in Tanzania found zoonotic transmission of *E. histolytica* (Deere et al., 2019). Enteric helminth eggs and parasitic cysts such as *Cryptosporidium* spp., *Giardia duodenalis*,

Cyclospora cayetanensis, *Entamoeba* spp., *Toxoplasma gondii*, *Balantioides coli*, *Cystoisospora belli* and *Enterocytozoon bieneusi* (Alemu et al., 2018; Amorós et al., 2010; El Bakri et al., 2020; Duedu et al., 2014; Nyarango et al,. 2008; Rodrigues et al., 2020; Utaaker et al., 2017) can adhere to vegetables and/or fruits and can transmit directly to humans, especially to those who consume raw vegetables (Al-Megrin, 2010; Rodrigues et al., 2020). Those parasites can also transmit to humans via water.

However, only a few studies have looked at transmission of organisms not associated with infectious diseases. The transmission of some eukaryotes does not have negative effects in humans. *Blastocystis* seems to be one of these eukaryotes. Its various possible transmission modes have been widely discussed: human-to-human, animal-to-human and environment-to-human. Household-based studies in the USA and Czech republic on *Blastocystis* have shown that the various subtypes were not shared between individuals (Scanlan et al., 2016; Lhotská et al., 2020). More recently, carriage studies in animal-to-human transmission have suggested that specific subtypes are zoonotic (Parkar et al., 2010; Alfellani et al., 2013b; Wang et al., 2014). For example, *Blastocystis* spp. in pigs is often transmitted to piggery staff. A previous study found that 13.9% of piggery staff carried *Blastocystis* sp. that was very similar to that of pigs, suggesting zoonosis (Yan et al., 2007, Wang et al., 2014) Similarly, the study of Thathaisong et al. (2003) in Thailand has found that high molecular similarity of *Blastocystis* sp. from humans and pigs and this might further confirm its zoonotic potential. Other studies have found that higher prevalence of *Blastocystis* in humans who were in close contact with poultry (Ramírez et al., 2014; Cian et al., 2017; Greige et al., 2018; Udonsom et al., 2018). Finally, sequences of *Blastocystis* sp. from children and monkeys living within the same area in Kathmandu, Nepal were highly similar (Yoshikawa et al., 2009).



Source Lee et al. (2012)

Figure 2.8 Life cycle of *Blastocystis* sp. with different ways of transmission

Most previous studies have focused on people living in urban areas of western countries. Studies on eukaryotic microbes and populations from Asian countries are extremely limited. Specifically, the study of occurrence, diversity, ecology, and transmission dynamics of eukaryotic microbes are completely unexplored

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CHAPTER 3

ENTAMOEBA CHIANGRAIENSIS N. SP. (AMOEBOZOA: ENTAMOEBIDAE) ISOLATED FROM THE GUT OF ASIAN SWAMP EEL (*MONOPTERUS ALBUS*) IN NORTHERN THAILAND[1]

Abstract

The genus *Entamoeba* comprises mostly gut parasites and commensals of invertebrate and vertebrate animals including humans. Herein, we report a new species of *Entamoeba* isolated from the gut of Asian swamp eels (*Monopterus albus*) in northern Thailand. Morphologically, the trophozoite is elongated and has a single prominent pseudopodium with no clear uroid. The trophozoite is actively motile, 30-50 μm in length and 9-13 μm in width. Observed cysts were uninucleate, ranging in size from 10-17.5 μm in diameter. Chromatin forms a fine, even lining along the inner nuclear membrane. Fine radial spokes join the karyosome to peripheral chromatin. Size, host and nucleus morphology set our organism apart from other members of the genus reported from fish. The SSU rRNA gene sequences of the new isolates are the first molecular data of an *Entamoeba* species from fish. Phylogenetic analysis places the new organism as sister to *Entamoeba invadens*. Based on the distinct morphology and SSU rRNA gene sequence we describe it as a new species, *Entamoeba chiangraiensis*.

Keywords: Archamoebae, Intestinal Protist, Morphology, Phylogeny, SSU rRNA

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3.1 Introduction

Entamoeba is a member of the Entamoebidae, a deep lineage within the Archamoebae (Pánek et al., 2016). *Entamoeba* species use pseudopodia for locomotion and lack flagella, a morphologically identifiable Golgi apparatus, peroxisomes, and canonical mitochondria (Loftus et al., 2005; Ptáčková et al., 2013). *Entamoeba* species have trophozoite and cyst stages. The latter may have one nucleus or as many as eight, each with peripheral chromatin prominently visible. Historically, cyst size and nuclear number and appearance, along with host range information, were considered taxonomically important features and used to identify and group species of *Entamoeba*. However, in recent years it has become obvious that morphological features alone are not sufficient to adequately discriminate species known to be genetically distinct (Clark et al., 2006; Stensvold et al., 2011). For example, morphology does not distinguish the morphologically identical *E. histolytica* and *E. dispar*, yet only the former is a human pathogen (Gonin et al., 2003; Fotedar et al., 2007a; Hooshyar et al., 2015). The advent of molecular tools has shed light on the taxonomic landscape of *Entamoeba* and clarified several issues associated not only with taxonomy, but also epidemiology and host range (Verweij et al., 2003; Fotedar et al., 2007b; García et al., 2014). Screening of fecal samples from a broad range of hosts using SSU rRNA gene primers has uncovered several new and distinct lineages of *Entamoeba*, indicating a richly diverse genus (Santos et al., 2010; Stensvold et al., 2011; Jacob et al., 2015). Much of this diversity had not been previously recognized.

Members of the genus *Entamoeba* generally inhabit the gastrointestinal tract of vertebrates and invertebrates, but they have also been observed within other protist cells (Ghosh, 1968; Stensvold et al., 2011; García et al., 2014; Shilton et al., 2018). Several *Entamoeba* species are parasitic, but commensals are more common (Hooshyar et al., 2015). Uniquely among members of the genus, *E. gingivalis* inhabits the human oral cavity (Ghabanchi et al., 2010; Luszczak et al., 2016; Maybodi et al., 2016). In addition, a few members of the genus have also been isolated from the environment (Clark & Diamond, 1997; Shiratori & Ishida, 2015).

Most *Entamoeba* gene sequences in public databases originate from species living in endothermic hosts, while relatively few derive from species living in ectotherms. To date, the latter hosts include amphibians, reptiles, and insects (Silberman et al., 1999; Garcia et al., 2014; Clark & Stensvold, 2015; Jacob et al., 2016; Kawano et al., 2017). Herein, we report a new species of *Entamoeba,* isolated from the gastrointestinal tract of the fish *Monopterus albus* (the Asian swamp eel) in Chiang Rai, Thailand. We examine its morphological features using light microscopy of living and stained specimens and provide the first SSU rRNA gene sequence of *Entamoeta* isolate an fish.

3.2 Methods

3.2.1 Sample Collection and Establishment of Culture

Two Asian swamp eels were purchased at a local market at Sanpong village, Phan district, Chiang Rai Province, northern Thailand. The eels were obtained at two separate times, in May and July 2018. Colonic contents were placed in modified (no mucin was added) LYSGM medium (Diamond, 1982) and incubated at room temperature (25-27 °C). After 24 hours, sediment was transferred to fresh medium and cells were subcultured every two weeks. The culture has been maintained since July 2018.

3.2.2 Light Microscopy and Staining

A wet mount of live amoebae was prepared and cells were observed using Nikon inverted light microscope. Trophozoites (n=10) and cysts (n=125 live; n=125 stained with iodine) were measured using the same microscope. For a more detailed view of the cells, iron hematoxylin staining was performed by the Diagnostic Parasitology Laboratory, London School of Hygiene and Tropical Medicine. Stained cells were observed with a Leica DMRB microscope fitted with a DFC 420 camera.

3.2.3 DNA extraction, amplification, purification and sequencing

Total genomic DNA was extracted from the culture using an AccuPrep® Genomic DNA Extraction Kit (Bioneer, South Korea, catalog No: K-3032) according to manufacturer’s specifications. Polymerase chain reaction (PCR) using the broad

specificity primers RD5 and RD3 was used to amplify almost the entire SSU rRNA gene (Table 3.1). Emerald Amp® GT PCR Master Mix for PCR reactions were obtained from TaKaRa Bio USA, Inc. Cycling conditions were as follows: initial denaturation at 94 °C for 3 min, followed by 40 cycles of: denaturation at 94 °C for 1.3 min, annealing at 60 °C for 1 min and extension at 72 °C for 2 min, ending with a final extension of 10 minutes at 72 °C.

The resulting PCR products were purified from gels with the GeneJET Gel Extraction Kit (Thermo Scientific; Wardmedic, Thailand) according to manufacturer's specifications. Samples were sequenced with RD5 and RD3 primers, along with ENTAM1, ENTAGENF and ENTAGENR (Table 3.1).

Table 3.1 Primers used to amplify and sequence *Entamoeba chiangraiensis*

Primer name	Primer sequence (5'—3')	References
RD5	ATCTGGTTGATCCTGCCAGT	
RD3	ATCCTTCCGCAGGTTCACCTAC	Clark et al. (2006)
ENTAGEN_F	ACTTCAGGGGGAGTATGGTCAC	
ENTAGEN_R	CAAGATGTCTAAGGGCATCACAG	Stensvold et al. (2011)
ENTAM1	GTTGATCCTGCCAGTATTATATG	Verweij et al. (2001)

3.2.4 Phylogenetic Analysis

The chromatogram quality of raw reads was checked individually with Sequencher software and ambiguous bases from the ends were removed. Sequences were combined into contigs and checked against the NCBI nr database, where they were identified as *Entamoeba*. A dataset was assembled including the newly derived sequences along with sequences spanning the breadth of molecular diversity of *Entamoeba*. In total, 90 sequences were used. Sequence alignment was performed on the EBI online platform (EMBL's European Bioinformatics Institute [EMBL-EBI], n.d.) using MAFFT v.7.394 (Katoh & Toh, 2010). Ambiguously aligned positions were removed using Trimal v.1.3 (Capella-Gutierrez et al., 2009) available on the online platform Phylemon 2.0 (n.d.).

After trimming 1,434 sites remained. Maximum likelihood analysis was conducted using RAxML v.8 (Stamatakis, 2006) on the online platform CIPRES Science Gateway (n.d.). For ML analysis, the general time reversible+Γ model of nucleotide substitution was employed as dictated by jModelTest v.2.1.10 using the Akaike criterion. Bootstrap support was computed from 1,000 bootstrap replicates.

3.3 Results

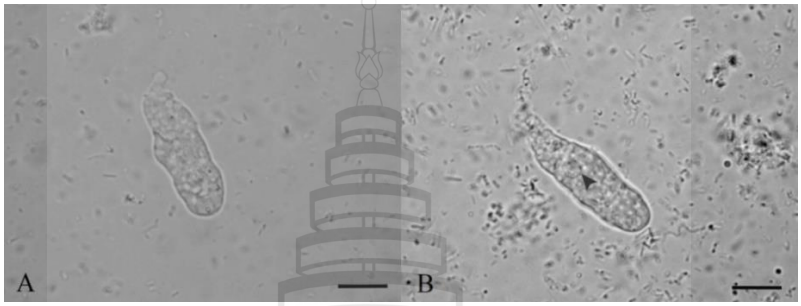
Culture, Light Microscopy and Phylogenetic Analysis

Colonic gut contents were inoculated into modified LYSGM, a medium widely used for xenic cultivation of *Entamoeba* species, and incubated at room temperature overnight. No live amoebae or cysts were observed in any tubes incubated at 37 °C, indicating that this species does not survive at that temperature.

The trophozoite of the amoeba is longer than it is wide (Fig. 3.1, Fig. 3.2C, 3.2D). Length is 40-50 μm (mean 44.31 μm) when the amoeba swims, but when it glides on the slide it ranges from 30-40 μm, while width ranges from 9-13 μm (mean 11.18 μm). The cell changes shape slowly while in motion and has a single prominent pseudopodium, while the posterior end is smooth with no obvious uroid (Fig. 3.1, Fig. 3.2C, 3.2D). The granuloplasm has multiple vesicles, while the hyaloplasm is narrow (Fig. 3.1A). Unstained spherical cysts range from 10.0-17.50 μm in diameter (mean 14.15 μm; ± 1.42 standard deviation; ± 0.13 standard error). Stained cysts range from 10.0-17.50 μm in diameter (mean 13.75 μm; ± 1.54 standard deviation; ± 0.14 standard error). All observed cysts in both live and stained samples were uninucleate (Figs 3.2A, 3.2B), with the exception of a single stained example where it looked like there were two nuclei. Large, prominent glycogen vacuoles were present in both live and stained cysts, indicating that all observed cysts were immature (Figs 3.2A, 3.2B). Therefore, we cannot state the number of nuclei per cyst definitively, as we were not able to observe mature cysts. Cysts have no distinctive appearance (Figs 3.2A, 3.2B).

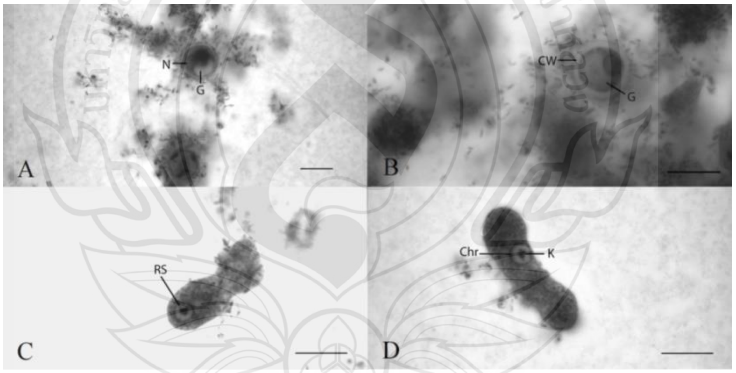
The size of the nucleus in both cysts and trophozoites ranges in diameter from 2.5-7.5 μm (mean 3.97 μm; ± 1.46 standard deviation; ± 0.13 standard error) and is generally found in the anterior half of the trophozoite. The trophozoite nucleus has a karyosome that has the appearance of a cluster of granules (Figs 3.2C, 3.2D).

Karyosome size is variable depending on how tightly the granules cluster. Chromatin forms a delicate, even lining along the inner membrane of the nucleus (Fig. 3.2D). Unlike many other *Entamoeba* species, there are no clearly visible clumps of peripheral chromatin. Radial spokes are present in the nucleus joining the karyosome to peripheral chromatin (Figs 3.2C).



Note Arrowhead indicates the nucleus. Scale bar = 25 µm

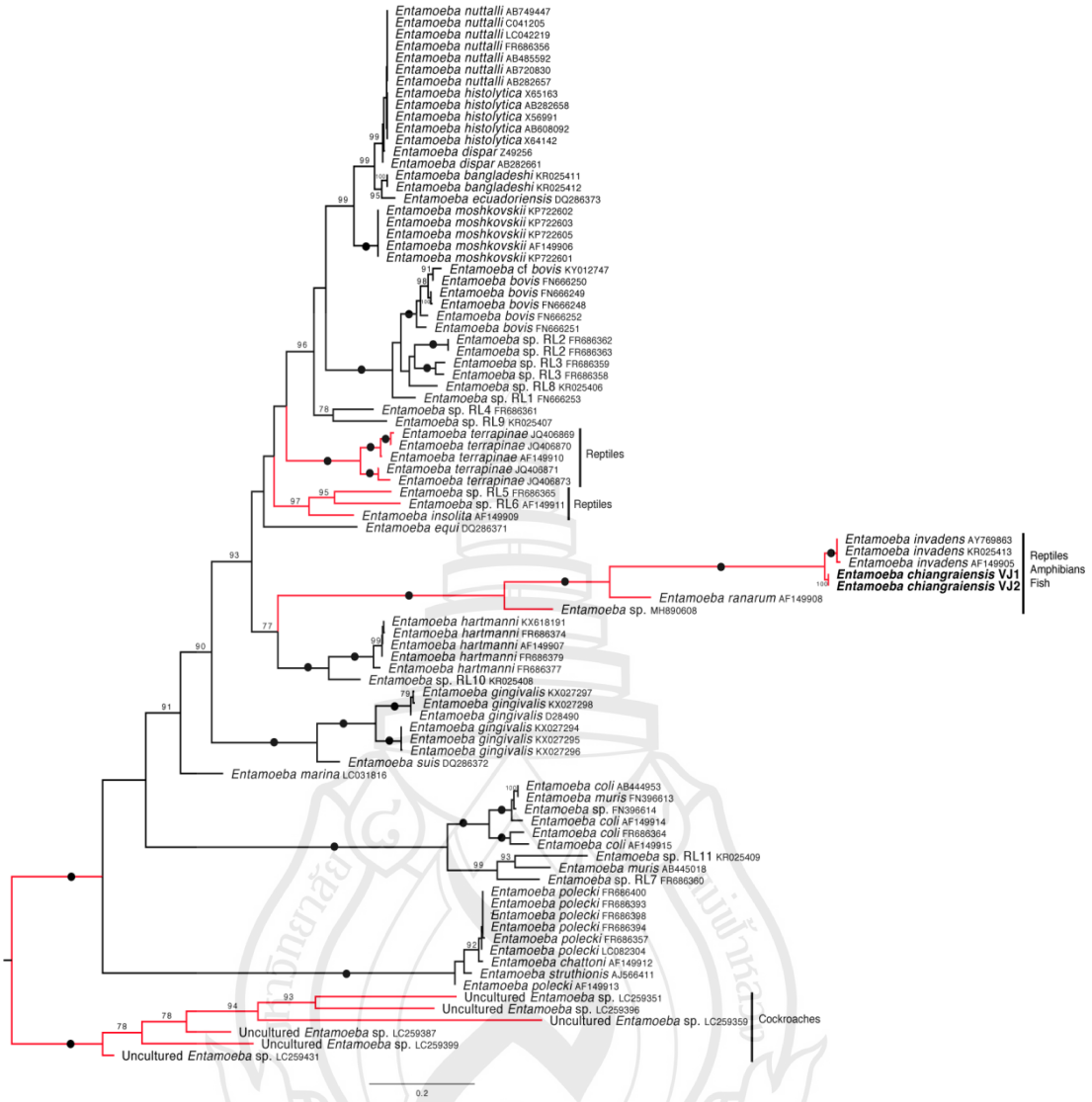
Figure 3.1 Light micrographs of living trophozoites of *Entamoeba chiangraiensis* n.sp



Note (A and B) Stained cysts. N, nucleus; G, glycogen vacuole; CW, cyst wall. (C and D) Stained trophozoites. RS, radial spokes connecting karyosome to peripheral chromatin; Chr, peripheral chromatin forming an even fine lining around nuclear membrane; K, karyosome consisting of granules. Scale bar = 10 µm.

Figure 3.2 Light micrographs of trophozoites and cysts stained with iron hematoxylin

The SSU rRNA gene sequences of the two isolates are nearly complete (1849 and 1856 bp). Both sequences have been deposited in GenBank under accession numbers MK652887 and MK652888. Overall topology of the phylogenetic tree is similar to previous studies (Jacob et al., 2015). The tree is artificially rooted to the clade containing the cockroach sequences. These were the earliest diverging *Entamoeba* sequences in the eukaryotic supergroup tree of Kawano et al. (2017). The new SSU rRNA gene sequences are sister to those from *E. invadens* and this relationship has maximum bootstrap support (Fig. 3.3). The genetic distance between the new sequences and *E. invadens* sequences ranges from 3.4%-3.8% (Table S1). All observed nucleotide differences (including insertion and deletion events) are taxon specific. Intraspecific genetic divergence for the new amoeba and *E. invadens* is 0% and 0.4%, respectively. These sister species are in a clade that also includes *E. ranarum* and an unnamed *Entamoeba* sp., both from amphibian hosts. All members of this clade have been isolated from ectothermic hosts. This clade also has maximum bootstrap support.



Note The tree is artificially rooted to cockroach-derived *Entamoeba* sequences. Newly generated sequences are depicted in bold lettering. Numerical values indicate bootstrap support. Only values above 70 are shown. Full circles represent maximum bootstrap support. Clades in red consist of sequences exclusively from ectothermic hosts

Figure 3.3 Maximum likelihood phylogenetic tree inferred from 90 SSUrRNA sequences and 1434 sites

Taxonomic Summary

Amoebozoa Lühe 1913, emend. Cavalier-Smith 1998

Archamoebae Cavalier-Smith 1983

Entamoebidae Chatton 1925, emend. Cavalier-Smith 1993

Entamoeba Casagrandi & Barbagallo 1895

Entamoeba chiangraiensis n. sp. Jinatham, Clark & Gentekaki 2019

Diagnosis: Amoeba inhabiting the gut of *Monopterus albus* (Asian swamp eel). Trophozoite is much longer than it is wide; length in motion is 30-50 μm, width 9-13 μm. Trailing end is smooth and devoid of visible uroid processes. Cysts are spherical, appearing smooth and thick-walled. Immature cysts have a single nucleus and a prominent glycogen vacuole, which often obscures the nucleus. Cyst diameter is 10.0-17.5 μm (mean 14.15 μm; ± 1.42 standard deviation; ± 0.13 standard error), nucleus 2.5-7.5 μm (mean 3.97 μm; ± 1.46 standard deviation; ± 0.13 standard error). There is a karyosome composed of granules. Chromatin is evenly distributed around the inner nuclear membrane, forming a thin, uniform lining. Radial spokes connect the karyosome to the peripheral chromatin.

Etymology: the epithet *chiangraiensis* refers to Chiang Rai province, Thailand, in which the organism was isolated

Host: *Monopterus albus*

Type location: isolated from the gut of Asian swamp eel, Sanpong, Phan, Chiang Rai, Thailand

Type material: permanent slide stained with iron-hematoxylin was deposited in the Smithsonian Museum under accession number USNM 1484171.

Type sequence: GenBank accession number MK652887

Table 3.2 Species of Entamoeba isolated from fish

Species	Host-salinity, location	# of cyst nuclei	Cyst diameter (µm)	References
Entamoeba chiangraiensis	Asian swamp eel (*Monopterus albus*), freshwater, Thailand	Uncertain	10.0-17.5	This report
Entamoeba ctenopharyngodoni	Carp, freshwater, China	1-4	7.8-10.4	Chen (1955)
Entamoeba gadi	Pollock (*Pollachius virens*), marine, USA	1-2	6.0-11.8	Bullock (1966)
Entamoeba molae	Ocean sunfish (*Mola mola*), marine, USA	1	Not observed	Noble and Noble (1966)
Entamoeba nezumia	Macrourid fish (*Nezumia bairdi*), marine, Greenland	1-4	7.7	Orias and Noble (1971)
Entamoeba pimelodi	Catfish (*Pimelodus clarias*), freshwater, Brazil	1	Not mentioned	Cunha and Penido (1926)
Entamoeba salpae	Fish (*Box salpa* syn. *Sarpa salpa*), marine, France	4	Not mentioned	Alexeieff (1912)
*Entamoeba synodontis**	Catfish (*Synodontis schall*), freshwater, Egypt	Uncertain	Uncertain	Imam *et al*. (1987)

Note Description is incomplete in the original te

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3.4 Discussion

Like all members of the genus *Entamoeba*, the new species has a nucleus with the characteristic “ring and dot” appearance corresponding to peripheral chromatin and central karyosome (Clark & Stensvold, 2015). *Entamoeba chiangraiensis* n.sp. was isolated twice from the Asian swamp eel, *Monopterus albus*, which inhabits rivers across Southeast Asia. Only a few species of *Entamoeba* from fish have been documented: four from marine hosts and three from freshwater (Table 3.2 and references therein). Molecular data for any of these species is absent.

Pathogenicity of the new species is unknown. Only a few species of *Entamoeba* are definitively pathogenic based on histology evidence. These are *E. histolytica*, a human pathogen, *E. nuttalli*, a pathogen of non-human primates, *E. invadens*, a reptile pathogen and *Entamoeba* sp., a toad pathogen (Clark & Stensvold, 2015; Shilton et al., 2018). Microscopic examination of *E. chiangraiensis* cells immediately after sample collection did not reveal ingestion of red blood cells, suggesting that the species is commensal rather than invasive. Nonetheless, to definitively determine pathogenicity further studies will be needed, including histology of infected fish to detect whether *E. chiangraiensis* invades host tissue.

We observed a single nucleus in cysts of the new species. However, the number of nuclei in mature cysts remains undetermined as cysts degenerated before reaching maturity. In the literature, the number of nuclei in cysts of Entamoebae from fish varies from one to four (Table 2 and references within). Species of *Entamoeba* from other ectothermic hosts commonly have four nucleated cysts, although octo-nucleated cysts have been observed in some reptiles, including *E. barreti* from a snapping turtle (Taliaferro & Holmes, 1924).

The host range of our and other species of *Entamoeba* from fish is unknown. We screened a number of fish inhabiting the same environment as the Asian swamp eel (Synbranchiformes) including: *Anabas* sp. (Anabatiformes, n=3), *Tilapia* sp. (Cichliformes, n-5), *Trichogaster* sp. (Anabatiformes, n=3), *Trachinocephalus* (Aulopiformes, n=2) and Siluriformes (Siluriformes, n=4). Our examination included both microscopy and a molecular survey using combinations of the primers described in the methods section. Intestinal contents from all fish were placed in the same culture medium in an attempt to grow amoebae. We were unable to find *Entamoeba* in any of the other hosts using any of the methods described. Although we tried to be as inclusive as possible in our screening, we cannot exclude the possibility that *E. changraiensis* might also inhabit the gut of fish that we have not examined. Host ranges of many *Entamoeba* species remain incompletely known, but they keep expanding. For instance, *E. coli* has traditionally been reported from humans and non-human primates, but is now known in rodents (Clark & Stensvold, 2015). Nonetheless, it seems likely that body temperature will pose a constraint on host range, as Entamoebae from ectotherms have not been found in endotherms and vice versa. *Entamoeba moshkovskii* is a notable exception, having been found in both reptiles and mammals (Garcia et al., 2014); it seems to be the only species of *Entamoeba* that has crossed the ectotherm/endotherm barrier. Within ectotherms, *Entamoeba* species show host specificity at the higher level of classification. Thus, reptilian isolates have never been isolated from amphibians and vice versa.

Entamoeba SSU rRNA gene sequences that have been detected exclusively in ectothermic hosts are diverse and dispersed across the phylogenetic tree, forming four distinct clades. The first clade comprises *E. chiangraiensis*, *E. invadens*, *E. ranarum*, and an unnamed *Entamoeba* sp. (MH890608) from a toad. The latter represents only the second amphibian-derived *Entamoeba* sequence. The SSU rRNA gene sequences from two eels sampled at two separate time points were identical, indicating low intra-specific diversity of this gene in *E. chiangraiensis*. This is similar to *E. invadens*, whose SSU rRNA gene sequences also display a high degree of genetic similarity, even when isolated from different hosts and from different countries (Jacob et al., 2015).

The new species groups together with *E. invadens*. When comparing their SSU rRNA sequences, the genetic distance is a little below 4%, almost four-fold than that between *E. histolytica* and *E. dispar*. The second clade contains several variants of *E. terrapinae* derived from aquatic turtles (Garcia et al., 2014). The third clade contains *Entamoeba insolita*, along with *Entamoeba* RL5 from tortoise and *Entamoeba* RL6 from iguana. These organisms are each represented by a single sequence (Silberman et al., 1999; Stensvold et al., 2011). Finally, the fourth clade consists of numerous sequences of *Entamoeba* from cockroaches (Kawano et al., 2017). In their study, Kawano et al. (2017) examined 186 cockroaches and found Entamoebae in 134. In their phylogenetic analyses, cockroach-derived sequences formed a distinct clade with nine separate groups within. This strongly hints at the presence of a vast diversity of *Entamoeba* that has yet to be uncovered. It seems likely that screening of additional hosts, especially ectotherms, will reveal an ever greater number of novel *Entamoeba* species.

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CHAPTER 4

PROTOTHECA BOVIS, A UNICELLUL ARACHLORO-PHYLLOUS TREBOUXIOPHYTE GREEN ALGA IN THE HEALTHY HUMAN INTESTINE[2]

Abstract

Prototheca species are non-photosynthetic trebouxiophyte algae ubiquitously distributed in nature and can be found in sewage and soil. This microbial eukaryote causes disease in farm and companion animals. Reports of human protothecosis are limited to immunocompromised individuals. *Prototheca* presence in stool of healthy individuals has been reported only rarely. There is an absence of detailed characterization of human *Prototheca* isolates. The aim of this study was to perform morphological and molecular characterization of *Prototheca* isolates obtained from human stool. *Prototheca* was isolated from fecal samples of four individuals living in a rural area in Thailand. A multiphasic approach combining bioimaging along with molecular and bioinformatics tools was used to characterize the four strains. Growth rate was tested using four media and three temperature conditions. Phylogenetic analysis using the small subunit ribosomal RNA (SSUrRNA) and cytochrome b (cytb) was also performed. Video stills capturing life stages and growth pattern of the organism were generated. Static and live microscopy demonstrated the various life stages of *Prototheca* and its major defining cellular characteristics. An optimized DNA extraction methodology that improves DNA yield is provided. Partial fragments of the SSUrRNA and cytb genes were obtained. Phylogenetic analysis placed all four

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strains within *Prototheca bovis*. More broadly, *Prototheca* was not monophyletic, but split into at least two distinct clades instead. The results represent the first molecular characterization of *Prototheca* in Thailand. In parallel, the study provides insight into transmission dynamics of the organism and potential caveats in estimating global prevalence of *Prototheca*. The findings in this study will spearhead further investigations on *Prototheca* occurrence in rural areas of both industrialized and developing nations.

Keywords: Gut Protists, Imaging, Infection, Phylogeny, *Prototheca*, Zoonosis

4.1 Introduction

Studies on human enteric organisms in Thailand have focused primarily on viral and bacterial pathogens, and parasitic worms, such as *Opisthorchis*, *Taenia* and *Enterobius* (Buathong et al., 2017; Sanpool et al., 2017; Tomanakan et al., 2020). With the exception of *Blastocystis*, *Cryptosporidium*, *Entamoeba*, and *Giardia*, other microbial eukaryotes are usually overlooked (Sannella et al., 2019; Tungtrongchitr et al., 2010; Udonsom et al., 2018; Yowang et al., 2018). One such example is *Prototheca*, a unicellular achlorophyllous trebouxiophyte green alga (Jagielski & Lagneau, 2007). *Prototheca* has been isolated from a broad range of animal species, including cats, dairy cattle, rats and swine (Jagielski et al., 2019; Scaccabarozzi et al., 2008). In dairy cattle, the organism causes mastitis (Osumi et al., 2008; Sobukawa et al., 2012; Zaini et al., 2012). Skin and soft tissue infections are seen in dogs and cats (Huth et al., 2015; Vince et al., 2014). Five species of *Prototheca* are known to infect humans and/or other animals: *P. blaschkeae, P. cutis, P. wickerhamii*, *P. ciferrii* (previously known as *P. zopfii* genotype 1) and *P. bovis* (previously known as *P. zopfii* genotype 2) (Pore et al., 1983; Roesler et al., 2006). The latter is of special interest as it has been found in both animals and humans. The disease caused by *Prototheca* is known as protothecosis (Hirose et al., 2018; Lass-Flörl & Mayr, 2007; Todd et al., 2012).

Prototheca bovis and *P. wickerhamii* are currently the two most common etiological agents of the disease (Buzzini et al., 2004; Lass-Flörl & Mayr, 2007; Thompson et al., 2019). *Prototheca* infections have been reported in Europe, Asia, North America and Africa (Ahrholdt et al., 2012). Nonetheless, the number of human cases is sparse: less than 200 cases of human protothecosis have been reported worldwide (De Kyvon et al., 2018; Todd et al., 2012). Human protothecosis has three main manifestations: cutaneous lesions, olecranon bursitis, and disseminated or systemic infection accompanied by varying symptoms (Pal et al., 2014). Cutaneous lesions are the most common manifestation of human protothecosis. *Prototheca* has only very rarely been isolated from the human intestine (Casal et al.,1983).

While performing a survey for eukaryotic microbes in a rural area of Thailand, we identified four potential cases of human intestinal protothecosis. Using a combination of tools from culturomics, cellular and molecular biology, as well as, phylogenetics we characterized the isolates as *Prototheca bovis*.

4.2 Methods

4.2.1 Ethics Statement

The Human Ethics Committee of Phramongkutklao College of Medicine approved collection of fecal samples from Thai volunteers (License approval number S053q/58).

4.2.2 Human Subjects and Sample Collection

Human volunteers were recruited as part of large-scale parasitological survey in Chachoengsao Province, Thailand. All volunteers were Thai nationals and lived in the province at the time of collection (Fig. 4.1). Fecal samples were randomly collected from 98 volunteers living in three villages (villages 11, 16 and 18), who did not have diarrhea at the time of collection. Sterile collection kits containing a plastic container, gauze and spatula were distributed to all volunteers. Small amounts of each fecal sample were introduced in two separate tubes containing HL-5 and LYSGM (Diamond, 1982) media. Upon transfer to the laboratory, all samples (98 tubes of HL-5 and 98 tubes of LYSGM) were incubated at 37°C.

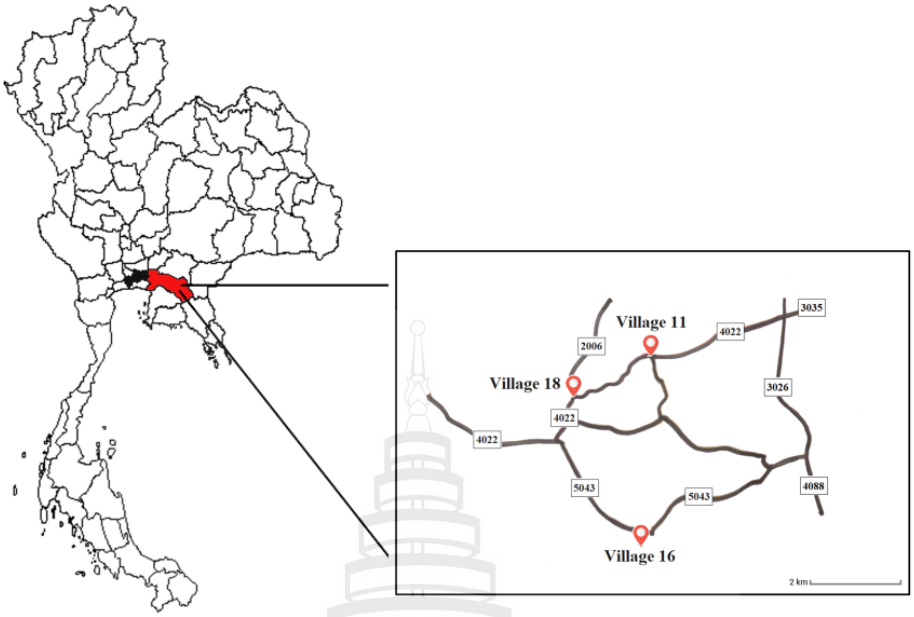


Figure 4.1 Sampling locations in villages 11, 16 and 18, Chachoengsao Province, Thailand

4.2.3 Culture Conditions

The cultures in HL-5 media and LYSGM were monitored daily using light microscopy starting from Day five post-inoculation. *Prototheca* cells were observed in samples from four volunteers in both media. One ml of *Prototheca* positive samples was sub-cultured in fresh media. After 24 hours, 10 µl of each *Prototheca* culture were streaked on Petri plates containing potato dextrose agar (PDA) and sealed with parafilm. Colonies appeared within 24 hours. A single colony was then picked from each of the four cultures and transferred into fresh PDA media. Cultures were also established in potato dextrose broth (PDB). Pure cultures of all four isolates were also established in blood agar, nutrient agar (NA) and malt extract agar (MEA) to examine macroscopic colony characteristics. The PDA plates were incubated at 25°C, 37°C, and 40°C for three days to observe growth pattern of *Prototheca* isolates. Photographs were taken on Day three.

Prototheca cells were counted using a haemocytometer and seeded at a density of 5000 cells per well of a 12 well plate (Greiner) containing 1 ml of PDB to generate video stills. The plate was mounted on a JuLi™Stage Real Time Cell History Recorder, inside an incubator at 37°C. The JuLi™Stage system was set to image the wells every minute until the cells in the field of view reached confluency using a 10x zoom lens. Image captures were then assembled into a video using the JuLi™Stage proprietary software. Image stills were taken using the snapshot function on the VLC media player (VideoLAN). Image editing was done using the Graphical Image Manipulation Program.

4.2.4 Microscopy

Fecal smears were prepared by diluting fecal matter with sterilized bottled drinking water. Micromorphological characteristics of *Prototheca* cells were observed by diluting PDB culture with bottled water. A Nikon 80i compound microscope equipped with a Nikon DS-Ri2 camera was used. Transmission electron microscopy (TEM) was carried out to characterize key morphological features in detail. Briefly, cells of *Prototheca* were centrifuged at 300 x g and resuspended in 2.5% glutaraldehyde, 100 mM sodium cacodylate buffer (pH 7.2) and fixed over night at 4°C. Samples were washed with cacodylate buffer twice for 10 minutes and then post-fixed in 1% osmium tetroxide in cacodylate buffer for one hour at room temperature. Samples were then washed twice in distilled water for 10 minutes and then dehydrated through a graded ethanol series of 50%, 70%, 90% at 10 minutes per step and then three times for 10 minutes in 100% ethanol. This was followed by two 10 minute incubations in propylene oxide, followed by 30 minutes in 50:50 propylene oxide:agar low viscosity (LV) resin. Samples were incubated twice for two hours in freshly prepared agar LV resin and then spun down in BEEM® capsules. The resin infiltrated samples were polymerised at 60°C for 24 hours. Sections of 70 nm were cut on a Leica EM UC7 ultramicrotome and collected on 400 mesh copper grids. Sections were counterstained in 4.5% uranyl acetate in 1% acetic acid for 45 minutes and Reynolds lead citrate for seven minutes. Sections were imaged in a Jeol 1230 Transmission Electron Microscope operated at 80 kV and images were captured with a Gatan One view digital camera.

4.2.5 DNA Extraction, PCR and Sequencing

Genomic DNA was extracted from pure cultures using Qiagen DNA stool mini kit (Qiagen, Hilden, Germany) with the following modification. Briefly, during the lysis step, 250mg of 0.5 mm zirconia beads and 20 µl of 10% SDS were added along with the lysis buffer provided by the kit. Polymerase chain reaction (PCR) was performed using EmeraldAmp GT PCR Master Mix (TaKaRa Bio USA, Inc.). Genomic DNA was also extracted from the faecal samples of the four *Prototheca* positive individuals. A fragment of 1550 bp of the small subunit ribosomal RNA (SSUrRNA) gene was amplified using DNA extracted from pure cultures with the NS1F: 5'-GTAGTCATATGCTTGTCTC-3' (White et al., 1990) and proto18S-4r: 5'-AGCACACCCAATCGGTAGGA-3' primers (Roesler et al., 2006). The PCR conditions were as follows: denaturation at 95°C for 3 min, 35 cycles with denaturation at 95°C for 2 min, annealing at 55°C for 90 sec, extension at 72°C for 2 minutes and a final extension at 72°C for 10 minutes. DNA extracted from the stool of *Prototheca* positive individuals was used in an attempt to amplify a fragment of 430 bp of the SSUrRNA using the proto18S-4f: 5'-GACATGGCGAGGATTGAC AGA-3' and proto18S-4r primers. The PCR conditions were as follows: denaturation at 95°C for 3 min, 35 cycles with denaturation at 95°C for 90 sec, annealing at 55°C for 1 min, extension at 72°C for 2 min and a final extension at 72°C for 10 minutes. All amplified products were purified with GeneJET Gel Extraction Kit (Thermo Scientific) and sent for bidirectional sequencing at U2Bio (Korea). A fragment of 600 bp of the cytb gene was amplified from DNA obtained from pure cultures using the cytb-F1: 5′-GyGTwGAACAyATTATGAGAG-3′ and cytb-R2: 5′-wACCCATA ArAArTACCATTCwGG-3′ primers (Jagielski et al., 2019). Sequences have been deposited in GenBank under the accession numbers MT920919, MW228085-87 (SSUrRNA) and MZ209407-MZ209410 (cytb).

4.2.6 Phylogenetic Analysis

The forward and reverse sequencing chromatograms were combined into contigs using CLC Main Workbench v.8 (Qiagen). The assembled sequences of SSUrRNA and cytb genes were used as queries to perform BLAST searches against GenBank to exclude contamination and to collect additional sequences of *Prototheca*

and other green algae. Two datasets were assembled, one for SSUrRNA and one for cytb. The SSUrRNA dataset contained 203 sequences spanning the diversity of trebouxiophyte algae and including reference and type sequences of *Prototheca* was assembled and aligned using MAFFT v.7.394 (Katoh & Toh, 2010). The cytb dataset contained 108 sequences. Ambiguous and poorly aligned regions were removed using Trimal v.1.3 (Capella-Gutiérrez, Silla-Martínez, & Gabaldón, 2009) available on the online platform Phylemon 2.0, (2011). Following trimming, 1117 and 597 sites remained in the SSUrRNA and cytb datasets, respectively. Maximum likelihood trees were constructed using RAxML v.8 (Stamatakis, 2006) available on CIPRES Science Gateway v. 3.3, (n.d.). The general time reversible + Γ model of nucleotide substitution was used. Bootstrap support was assessed from 1000 bootstrap replicates.

4.3 Results

4.3.1 Isolation and Culturing of *Prototheca bovis* from Four Human Stool Samples

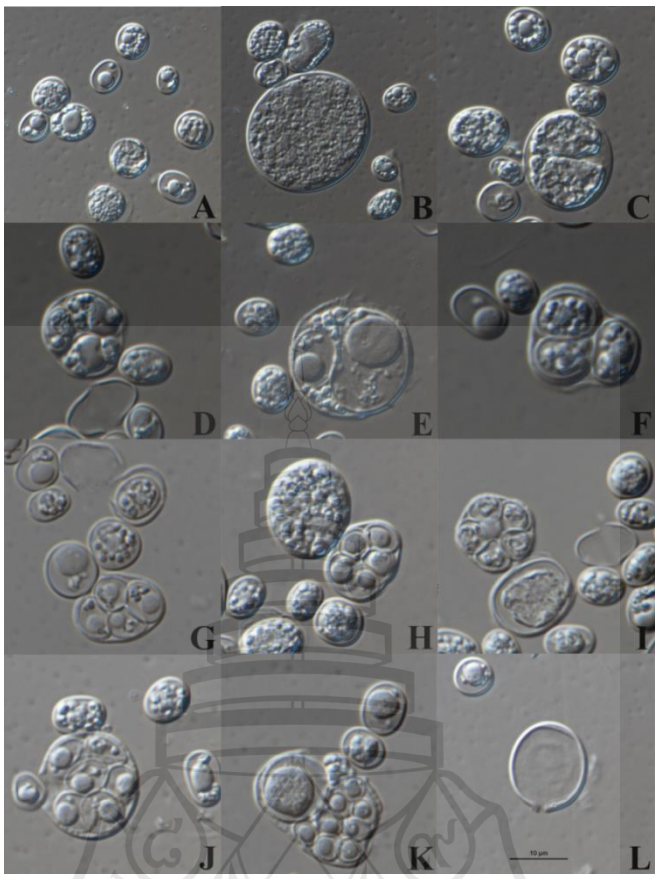
Culture media containing fecal samples of 98 volunteers were examined using a compound microscope. *Prototheca* was found in the stool of four volunteers. Fecal smears of the *Prototheca* positive stool samples provided additional confirmation of its presence (Sup. Fig. 4.1). Colonies grew on all four media (PDA, MEA, NA and blood agar). Cells appeared within 12 hours and dense colonies appeared on the nutrient-rich PDA, MEA and blood agar plates, while colonies were smaller and not as dense in the nutrient-poor NA (Sup. Fig. 4.2). Colonies were yeast-like and with smooth edges. In PDA, MEA and blood agar, colonies were pale white to cream white, 2 to 3 mm in diameter, while those in NA were one third of the size at the same time of incubation (Day 3). *Prototheca* colonies were also incubated at different temperatures to observe growth. At 25°C, colonies of *Prototheca* on PDA were smaller than those at 37°C and 40°C on day 3 (Sup. Fig 4.3).

4.3.2 *Prototheca bovis* Growth Pattern

The algal growth, maturation and division were recorded live. Cells seeded in PDB media were monitored until the field of view was confluent. Upon maturation, cells (sporangia) ruptured to yield eight daughter cells (sporangiospores) (Sup. File 4.1). Under the growth conditions used herein, newly divided daughter cells reached maturity and divided anew in five and a half hours (Sup. File 4.1). A crowded environment appeared to have an effect on cell size, as when the field of view reached confluency, most of the newly divided cells were smaller in size compared to cells of previous generations.

4.3.3 Microscopic Investigations Highlights Basic Cell Features

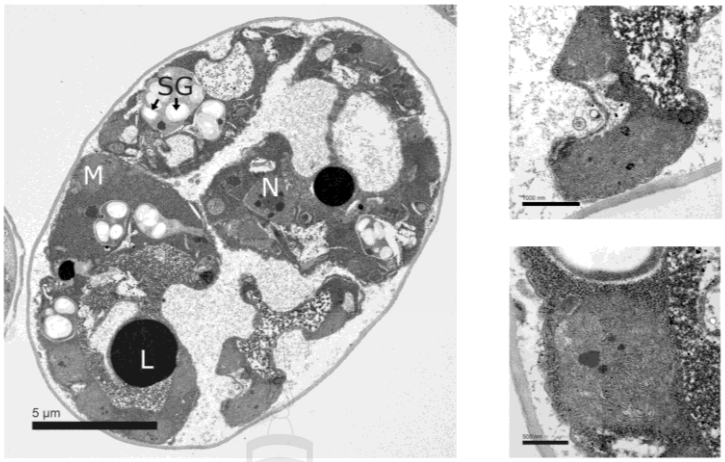
All four isolates formed sporangia seen at various stages of multiplication containing two, four or eight sporangiospores. Sporangia with more than eight sporangiospores were not observed at any point. Sporangiospores arranged in morulae (flower petal formation) were commonly observed (Fig. 4.2). Sporangiospores were 7.0-16.0 µm long x 6.0-12.5 µm wide. Thick septa were present between sporangiospores within sporangia. The thick cell wall characteristic of *Prototheca* was clearly visible.



Note *Prototheca* sp. sporangiospores (A); sporangium just before first cell division, granular content clearly visible (B); sporangia at various stages of division and released sporangiospores (C-I); sporangiospore exiting bursting sporangium (J); mature sporangium containing eight sporangiospores (K); Remnant cell wall of a bursting sporangium (L). Scale bar: 10 μm

Figure 4.2 Details of *Prototheca* sp. cells incubated on PDA medium at 37^{o}C for 72 hours

Transmission electron microscopy was used to obtain high-resolution images of *Prototheca* cells. Various life stages of *Prototheca* and its subcellular organelles were observed (Fig. 4.3). These included a cell membrane surrounded by a thick cell wall, canonical mitochondria (numerous cristae), remnant plastids with double membranes, filled with starch granules and dense lipid droplets of various sizes dispersed in the cell (Fig. 4.3).



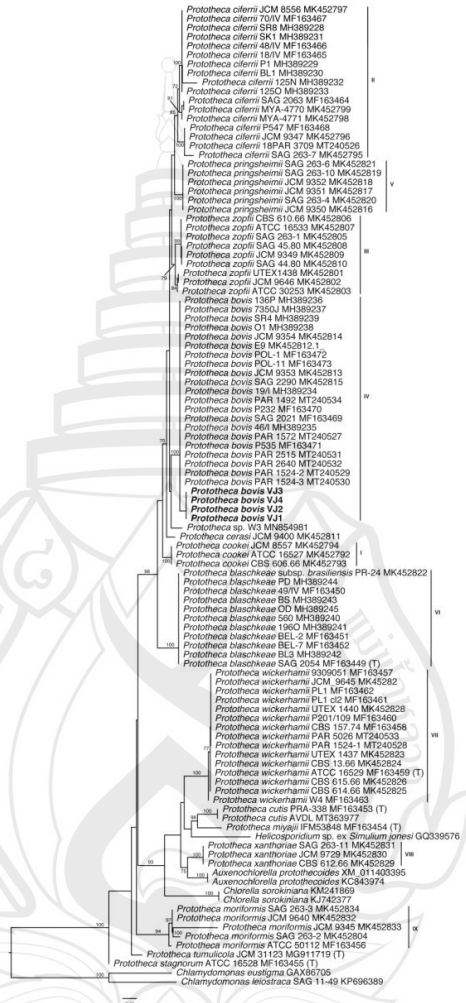
Note Lipid droplets (L), nucleus (N), mitochondria (M), starch granules (SG) and a thick cell wall (Panel A). Close up of mitochondria located near the cell walls (Panels B and C)

Figure 4.3 Transmission electron microscopy depicting structural features commonly associated with *Prototheca* species

4.3.4 Sequence and Phylogenetic Analyses

Prototheca sequences were successfully obtained only for the fragments amplified from the DNA obtained from pure cultures. The sequences from DNA extracted from stool samples were from edible plants. The newly generated SSUrRNA sequences were identical to each other. Two separate phylogenetic trees were inferred from SSUrRNA (Sup. Fig 4) and cytb (Fig. 4) gene sequences. In the SSUrRNA tree, *Prototheca* was not monophyletic, as it split into three clades with *Helicosporidium* grouping within. These clades were: (1) a clade containing most *Prototheca* species; (2) a clade containing *P. miyajii* and *P. cutis*, which grouped as sister to *Helicosporidium*, albeit with low support; and (3) the *P. xanthoriae* clade, which grouped as sister to *Auxenochlorella*. The latter clade contained two *P. xanthoriae* sequences, which grouped separately from the type sequence of this species, which groups within clade 1. The four newly identified strains nested within *P. bovis* sequences along with a single sequence of *P. xanthoriae* in clade 1. In the cytb tree, *Prototheca* was also not monophyletic with strains of *Chlorella*, *Helicosporidium* and *Auxenochlorella* grouping within.

The *Prototheca* sequences were distributed into the same nine previously defined clusters (I-IX), with each cluster representing a separate species (Jagielski et al., 2019). The newly generated sequences grouped with *P. bovis*, thus we have designated them as such.



Note Numerical values at nodes represent maximum likelihood bootstrap support. Only values above 70 are shown. New sequences are presented in bold letters. Sequences from type specimens are represented with a (T). Roman numerals (I-IX) represent *Prototheca* species clusters as those are defined in Jaglieski et al. (2019).

Figure 4.4 Maximum likelihood phylogeny of cytochrome b inferred from 108 sequences and 597 sites

4.4 Discussion

Prototheca is likely transmitted to humans by an animal or environmental reservoir even though these sources have yet to be identified (Pore et al., 1983). Human protothecosis manifests as localized infection of the skin, olecranon bursitis and disseminated infection (Chou et al., 2014; De Kyvon et al., 2018; Todd et al., 2012). It has been proposed that immunocompromised individuals and those with underlying conditions are more susceptible to the disease (Khan et al., 2018; Lanotte et al., 2019). The two most common species of *Prototheca* causing human infection are *P. bovis* and *P. wickerhamii*. The latter is the principal etiological agent of human protothecosis, whereas *P. bovis* commonly causes disease in other animals (Jagielski et al., 2019; Pal et al., 2014). Workers in rice paddies, fishermen, farmers, handlers of raw seafood, and aquarium staff are at risk of exposure to *Prototheca* (Pore et al., 1983). Nonetheless, there are only a few studies from countries where these jobs are common, including South and Southeast Asian countries. In Thailand, *Prototheca* has been reported only rarely (Pongsripian et al., 2000; Thianprasit et al., 1983).

Traditionally, protothecosis diagnosis has focused on microscopic observation and physiological/biochemical tests of the isolated organism or direct examination of affected tissues (De Kyvon et al., 2018). Nonetheless, neither of these methods can accurately identify *Prototheca* to the species level. Molecular characterization using SSUrRNA and *cytb* genes has greatly facilitated species identification (Jagielski et al., 2019; Kunthiphun et al., 2019). Herein we isolated four strains of *Prototheca* from fecal samples of Thai volunteers living in the rural area of Chacheongsao. Phylogenetic analysis of the SSUrRNA and cytb genes placed all four isolates in the *P. bovis* clade (Sup. Fig. 4.4, Fig. 4.4). This species is associated with bovine mastitis (in the form of persistent udder inflammation) and infections of companion animals (Marques et al., 2008; Roesler et al., 2016; Stenner et al., 2007; Thompson et al., 2009). Nevertheless, there have been occasional reports of clinical cases of human protothecosis involving *P. bovis* (Seok et al., 2013; Severgnini et al., 2018; Takano et al., 2014; Todd et al., 2018). This species was previously designated as *P. zopfii*

genotype 2 and was recently elevated to species level (Jagielski et al., 2019). Given that finding *Prototheca* in human stool is extremely rare, its occurrence in four individuals raises questions about its original source, distribution and transmission.

Pinpointing the source of *Prototheca* is challenging, as human-to-human transmission is unknown (Lass-Flörl & Mayr, 2007). Herein, volunteers positive for *Prototheca* lived in four separate households distributed in two different villages 15 km apart. Participants in this study spend extended periods of time in rice fields and have frequent contact with ruminants and poultry, whose dung is used to fertilize their vegetable gardens. The three villages' water source comprises groundwater stored in tanks composed of layers of sand and gravel filters. Rice fields, dung and groundwater constitute habitats from which *Prototheca* has been previously isolated (Pore et al., 1983). Hence, the alga likely came from one or all of the aforementioned sources, though it is not possible to confidently identify the source. Given these uncertainties, a comprehensive One Health approach that includes humans, other animals and the environment in all three villages is needed in the future.

Remarkably, little is known regarding pathogenesis and virulence of *Prototheca*. Many studies focus on infections of immunocompromised hosts and discovery of the organism is often incidental (Khan et al., 2018). Notwithstanding, its ability to survive passage through the gastrointestinal tract has been previously discussed (Pore et al., 1983). In this study, *P. bovis* was detected in the stool of four individuals that exhibited no diarrhea. Smears of stored fecal samples from all *Prototheca* positive volunteers revealed the presence of organisms, some of which were dividing (see Sup. Fig. 4.1). This finding suggests that the organism remained in the host long enough to undergo cell division. It is currently not feasible to determine whether infection was established as samples were collected during a large-scale survey, where we checked only the diarrhea status. At the same time, one cannot exclude the possibility of *P. bovis* asymptomatically colonizing the hosts.

The rarity of protothecosis is a recurrent theme of literature reviews (Shave et al., 2021; Todd et al., 2012). Our study suggests methodological caveats as a possible cause. At the onset of the experiments, DNA yield from the newly established pure cultures of *P. bovis* was disproportionately low given the large number of cells used for the extraction. Direct microscopic observation of cells right after lysis, revealed

that the vast majority of them failed to lyse. This is not surprising given the thick cell wall of *Prototheca* (see Fig. 4.3). After adapting various protocols, we found that using zirconia beads (0.5 mm) along with SDS (10%) improved DNA yield substantially. Microscopic examination showed that more than half of the cells had ruptured after using this lysis method. Given the difficulty of cell rupture we started to suspect that *Prototheca* cells in stool might not be disrupted either when using stool DNA extraction methods. To further examine this, we went back to the DNA obtained from the four original stool samples of *Prototheca* positive volunteers. To our surprise, attempts to amplify and sequence SSUrRNA and cytb gene fragments from these DNA samples only yielded sequences of edible plants. Collective consideration of these data strongly suggests that the alga remained intact during DNA extraction from stool samples. If this is the case, then it is highly likely that prevalence of *Prototheca* is consistently underestimated and not considered in surveys of fecal and/or environmental eukaryotic diversity.

Herein we performed molecular characterization of *Prototheca* from the stool of four volunteers in Thailand. Based on the potential methodological caveats discussed above, we speculate that *Prototheca* prevalence might be underestimated in human hosts. During this study, it became evident that there is a need for further studies to determine whether the organism is transient, temporary/long-term colonizer or a pathogen. Thus, there is an urgent need to elucidate aspects of the biology and life cycle of *Prototheca*. In that vein, we outline a toolkit of techniques that can be used to study the cell biology and characterize specific components of *Prototheca* in the future. Such approaches comprise a significant step towards developing this organism as a model to understand various aspects of its pathogenicity and opportunistic nature.

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CHAPTER 5

BLASTOCYSTIS ONE HEALTH APPROACH IN A RURAL COMMUNITY OF NORTHERN THAILAND: PREVALENCE, SUBTYPES AND NOVEL TRANSMISSION ROUTES[3]

Abstract

Blastocystis is the most commonly found eukaryote in the gut of humans and other animals. This protist is extremely heterogeneous genetically and is classified into 28 subtypes (STs) based on the small subunit ribosomal RNA (*SSU* rRNA) gene. Numerous studies exist on prevalence of the organism, which usually focus on either humans or animals or the environment, while only a handful investigates all three sources simultaneously. Consequently, understanding of *Blastocystis* transmission dynamics remains inadequate. Our aim was to explore *Blastocystis* under the One Health perspective using a rural community in northern Thailand as our study area. We surveyed human, other animal and environmental samples using both morphological and molecular approaches. Prevalence rates of *Blastocystis* were 73% in human hosts (n=45), 100% in non-human hosts (n=44) and 91% in environmental samples (n=35). Overall, ten subtypes were identified (ST1, ST2, ST3, ST4 ST5, ST6, ST7, ST10, ST23 and ST26), eight of which were detected in humans (ST1, ST2, ST3, ST4, ST5, ST7, ST10 and ST23), three in other animals (ST6, ST7 and ST23), while seven (ST1, ST3, ST6, ST7, ST10, ST23 and ST26) were found in the environment. In our investigation of transmission dynamics, we assessed various groupings both at the household and community level. Given the overall high prevalence rate, transmission amongst humans and between animals and humans

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are not as frequent as expected with only two subtypes being shared. This raises questions on the role of the environment on transmission of *Blastocystis*. Water and soil comprise the main reservoirs of the various subtypes in this community. Five subtypes are shared between humans and the environment, while three overlap between the latter and animal hosts. We propose soil as a novel route of transmission, which should be considered in future investigations. This study provides a thorough One Health perspective on *Blastocystis*. Using this type of approach advances our understanding on occurrence, diversity, ecology and transmission dynamics of this poorly understood, yet frequent gut resident.

Keywords: Asymptomatic Hosts, *Blastocystis*, Environmental transmission, One Health, Rural Community, Thailand

5.1 Introduction

Blastocystis is the most ubiquitous protist inhabiting the gastro- intestinal tract of human and other animal hosts (Roberts et al., 2013; Beghini et al., 2017; Stensvold & van der Giezen, 2018). Historically, diagnosis of *Blastocystis* has been based on light microscopy of fecal smears or *in vitro* cultures. The organism has four morphological forms: vacuolar, granular, amoeboid, and cyst (Tan, 2008; Parija et al., 2013). The lack of distinct morphological features had, in the past, blurred the extent of *Blastocystis* diversity. Based on the genetic heterogeneity of the small subunit ribosomal RNA (*SSU* rRNA), *Blastocystis* is currently divided into at least 28 subtypes (STs) consisting of ST1-ST17, ST21, and ST23-ST32, all of which have been found in mammalian and avian hosts and are likely separate species (Stensvold, Alfellani & Clark, 2012; Alfellani, Taner-Mulla et al., 2013; Zhao et al., 2017, Maloney et al., 2020; Stensvold & Clark, 2020; Maloney, da Cunna et al., 2021; Maloney, Jang et al., 2021; Higuera et al., 2021). Several genetically distinct *Blastocystis* lineages have also been identified in amphibian, insect and reptilian hosts, however these are not part of the subtyping nomenclature as yet (Yoshikawa et al., 2007; Yoshikawa, 2016).

Despite earlier assumptions, subtypes do not seem to be host specific. So far, ST1-ST9 and ST12 have been reported in humans along with a single instance of ST10, ST14 and ST16 (Stensvold & Clark, 2016; Khaled et al., 2020; Osorio-Pulgarin et al., 2021). The most frequently encountered subtypes in humans are ST1-ST4, with the latter being most often reported in Europe (Deng et al., 2019; Jiménez et al., 2019; Stensvold, Tan & Clark, 2020). Nonetheless, the subtypes reported in humans have also been found in non-human hosts. For example, ST1 and ST3 have been identified from pigs, while ST4 is dominant in rodents (Yoshikawa, Abe & Wu, 2004; Stensvold, Alfellani et al., 2009; Alfellani, Stensvold et al., 2013; Wang et al., 2018; Betts et al., 2021).

After more than a century of research, the pathogenicity of *Blastocystis* remains questionable. Its presence in sufferers of chronic gastrointestinal illnesses including irritable bowel syndrome and inflammatory bowel disease has led to

speculations about possible links to these disease states (Dogruman-Al, Kustimur et al., 2009; Tan et al., 2010; Poirier et al., 2012; Cifre et al., 2018; Kesuma et al., 2019; Peña et al., 2020; Shirvani et al., 2020). However, recent studies have increasingly shown that *Blastocystis* is a frequent and stable inhabitant in the gut of hosts without gastrointestinal symptoms (Scanlan et al., 2014; Mirjalali et al., 2017; Riabi et al., 2018; Yowang et al., 2018; Kataki, Tavalla & Beiromvand, 2019; Lhotská et al., 2020; Padukone et al., 2021). In parallel, this protist has been linked with increased bacterial richness and diversity in the human gut (Audebert et al., 2016; Chabé et al., 2017; Laforest-Lapointe & Arrieta 2018; Tito et al., 2019; Deng et al., 2021). Therefore, a plethora of researchers now consider *Blastocystis* as a commensal rather than a pathogen.

Understanding various aspects of *Blastocystis* epidemiology will contribute significantly towards determining its pathogenicity and/or virulence of the various subtypes. To that end, elucidating routes of transmission and contributions of various sources to these routes is essential. The human-to-human, zoonotic, and waterborne transmission routes have been explored in relation to *Blastocystis* prevalence (Eroglu & Koltas, 2010; Alfellani, Taner-Mulla et al., 2013; Maloney et al., 2019). Occurrence of certain subtypes in both human and other animal hosts has led to the hypothesis that these are subtypes of zoonotic potential. For instance, ST5, typically found in pigs, and ST6, ST7 typical subtypes of avian hosts, have also been found in humans that handle them extensively (Wang et al., 2014; Griege et al., 2018). Transmission of ST8 has also been noted between non-human primates and their human zookeepers (Stensvold, Alfellani et al., 2009). Waterborne transmission of *Blastocystis* has been long recognized (Li et al., 2012; Andersen & Stensvold, 2015). For instance, ST1 was identified in the water supply of a rural community in central Thailand and schoolchildren that consumed it (Leelayoova et al., 2008) and in untreated drinking water in Peninsular Malaysia (Anuar et al., 2013). Nonetheless, only scant studies simultaneously consider the contribution of more than one source to *Blastocystis* transmission.

In general, investigating transmission dynamics requires conditions that allow for uninterrupted cycling of an organism in a community. As such, developing countries comprise ideal areas to undertake these types of approaches. Herein, we undertook a One Health approach to examine *Blastocystis* epidemiology in a rural community of northern Thailand. We collected samples from humans, other animals and the environment and screened them for presence of *Blastocystis*. Data were analyzed at singular and community levels. We identified water and soil as the primary contributing sources to *Blastocystis* transmission routes in this particular community. These findings provide a multi-layered understanding of the transmission dynamics (spreading and cycling) of this controversial protist.

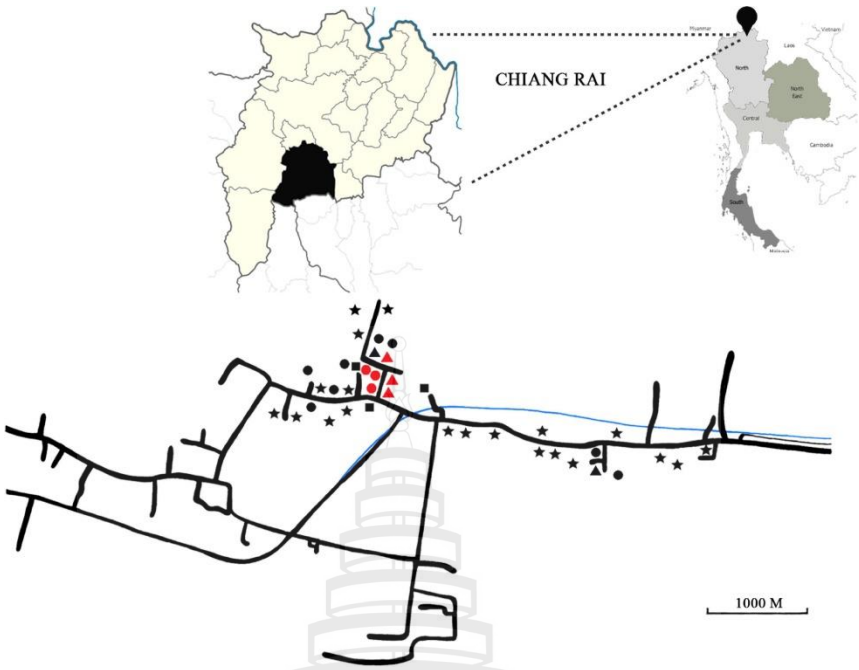
5.2 Methods

5.2.1 Ethics Statement

The ethics committee of Mae Fah Luang University approved collection of human and animal samples used in this study (human license approval number REH60103 and animal license approval number AR01/62). Ethical rules were in accordance to the Declaration of Helsinki. Data were strictly anonymized and each sample was assigned an individual barcode.

5.2.2 Study Area

This study took place in a century-old rural community of 500 inhabitants in Chiang Rai Province, Thailand, between 2018 and 2019. The province is located in northern Thailand and borders Myanmar (Figure 5.1). The area of study is located across a river and villagers feed mainly on fish, vegetables and sticky rice. All residents are Thai nationals with no travel history of going abroad. There has been no immigration in the community for the last 20 years. The distance from the closest urban center is 20 km.



Note Top left panel: Close-up of Chiang Rai Province (in pale yellow) and the district where sampling took place (in black). Bottom panel: Detail of area of collection used in this study. Geometrical shapes represent households. Stars: Only human stool was collected. Triangles: human stool, animal stool and water were collected. Squares: Human and animal stool was collected. Circles: Human stool and water were collected Red shapes indicate households, where stool samples were collected from all members.

Figure 5.1 Top right panel: Map of Thailand. Black pin has been placed on Chiang Rai Province

5.2.3 Sample Collection

A summary of the methodology used is provided in Figure 5.2.

Human fecal samples. Fecal samples were collected from 45 Thai adults. Each participant was provided with a sterile sampling kit containing collection container, gauze and spatula. Volunteers did not suffer from gastrointestinal diseases and had no gastrointestinal symptoms at the time of sampling. Samples were collected from 39 households, six of which housed families. A family was defined as a group of at least two people living under the same roof.

Other animal fecal samples. Fecal samples were collected from 44 animals including chickens, buffalo and pigs. These animals are representative of the livestock present in the community. Several stray dogs wander freely around the community and cannot be assigned to an owner, hence these were not sampled. The animals from which samples were obtained could be traced to specific households. Chickens (n=34) were free-range and lived in tight proximity to the household, while buffalo (n=4) and pigs (n=6) were housed further away from the house. Animals did not have diarrhea or blood in their stool at the time of sampling.

Water samples. Water in the area was surveyed to investigate the possibility of an environmental reservoir of *Blastocystis*. A total of 28 water samples were collected, 17 of which were from rain collection vessels (Supplementary Material, Figure S1). These are cement containers (~ 2 m in height) present in most houses. Most of the water comes from direct rain run-off from the roof of the house. A pipe directly connects the roof to the container. Cotton plugs serve as filters to catch leaves and wood debris. A lid rests over the containers most of the time. This water is used for drinking and cooking. The containers are washed once a year during the dry season. A tap is located at the bottom of each container. One L of water was taken from the tap of each container. Water from all containers was turbid at visual inspection.

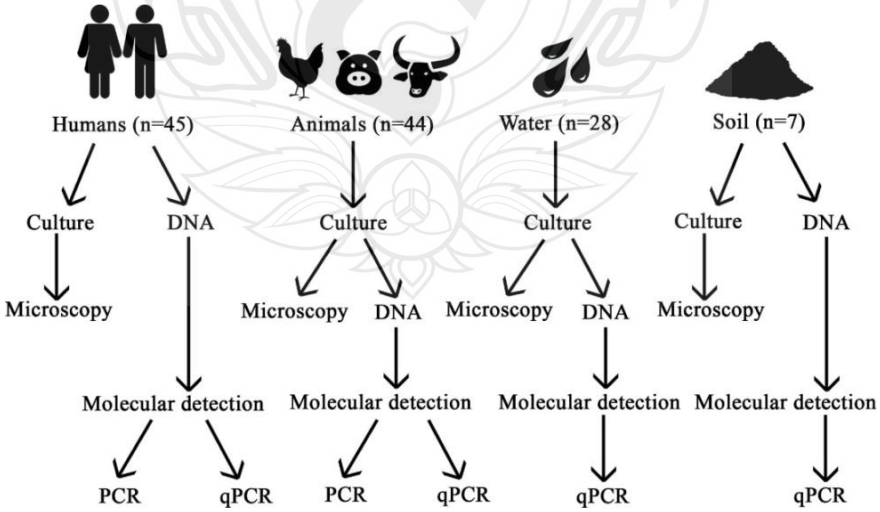


Figure 5.2 Flow chart of the methodology used in this study

Three samples of one L each were collected from the single community supply water-dispensing machine (Supplementary Material, Figure S2). The water comes from a waterfall, filtered and dispensed from the machine into 10 L containers. This water is used for drinking and cooking. The containers are washed with pressurized water regularly. We sampled three of those containers.

Two samples of one L each were collected from the water treatment facility, from which water is distributed to every household through pipes (Supplementary Material, Figure S3). The water is taken directly from the river and occasionally treated with chlorine. This water is used for bathing and watering plants (edible and non-edible).

One sample was collected from the community water tower (Supplementary Material, Figure S3C). The water from the tower comes from the water treatment facility. The bottom of each tower is lined with layers of sand and gravel, which serve as additional filters. Villagers can get their water through a tap located at the bottom of the tower. The water is used mostly for cooking and bathing and rarely for drinking. A one L sample was taken from the tap and the water was turbid at visual inspection.

Two samples of one L each were collected from the river stream, which is densely grown with morning glory plants (*Ipomoea aquatica Forssk*) and eaten raw or cooked (Supplementary Material, Figure S4A). At the time of collection, water depth was 20 cm. Water was collected from the middle of the stream and was very turbid at visual inspection.

A single sample was collected from an artificial pond with soil sediment. The pond is used for fish farming (Supplementary Material, Figure S4B). A one L sample was taken from the shallow end of the pond. Sample was very turbid at visual inspection.

Two samples were taken from a cement container, which is used for short term holding of live fish and amphibians (Supplementary Material, Figure S4C). Occasionally, the water from the pond and the cement container is used for watering gardens. A one L sample was collected and was slightly turbid at visual inspection.

Soil samples. Seven soil samples were collected from a depth of no more than 5 cm using sterile spoons. Each sample consisted of 2-3 g of soil. Four of these came from four separate vegetable gardens (Supplementary Material, Figure S5A-D). Three of the gardens were field plots, while one comprised of pots. One soil sample came from an ephemeral stream, where the local herb Plu Kaow grows (*Houttuynia cordata Thunb*). Villagers use this herb extensively (raw or cooked) for vegetable side dishes accompanying raw meat. The stream was void of water, but muddy at the time of collection. One sample was also gathered from river sediment. One soil sample was picked from the riverbank. Both the river and the riverbank are overgrown with morning glory (Supplementary Material, Figure S6).

5.2.4 *Blastocystis* Cultures

For human and other animal fecal samples, approximately 200 mg of freshly collected feces were placed in LYSGM (Diamond, 1982) containing 10% horse serum. Water samples were left to sit for three hours on a flat surface. Subsequently, 2-4 ml was taken from the bottom of each sample and placed in LYSGM. Soil samples were thoroughly mixed and 100 mg placed in LYSGM. Tubes were incubated at 37 °C for 48-72 hrs and screened for *Blastocystis* using light microscopy.

5.2.5 Genomic DNA Extraction

Human and other animal fecal samples. In the case of human samples, DNA was extracted from feces using 200 mg. DNA from animal samples was extracted prior to the first passage of culture using 250 mL of sediment from each sample. The Qiagen DNA stool minikit (Qiagen, Hilden, Germany) was used according to manufacturer's protocol.

Water samples. DNA was extracted from 250 mL of culture sediment using AccuPrep® Genomic DNA Extraction Kit following the manufacturer's protocol.

Soil samples. DNA from soil was directly extracted from 200 mg of soil using PowerSoil® DNA Isolation Kit (Carlsbad, CA USA) according to manufacturer's protocol.

5.2.6 *Blastocystis* Detection

Three approaches were used to detect *Blastocystis* from human samples: microscopy following culturing in LYSGM, conventional PCR and qPCR (Figure 2). For the rest of the samples only microscopy and qPCR were used.

5.2.7 Polymerase Chain Reaction (PCR) and Sequencing

The broad specificity primer pair RD3 5′ GGGATCCTGATCCTTCCGCA GGTTCACCTAC-3′ and RD5 5′-GGAAGCTTATCTGGTTGATCCTGCCAGTA-3′ (Clark, 1997) was used for the first PCR reactions with the following conditions: initial denaturation for 3 min at 94 °C, 35 cycles at 94 °C for 1 min, annealing 60 °C for 1 min, and extension at 72 °C for 100 sec, with a final elongation step at 72 °C for 7 min. A 600 bp fragment of *SSU* rRNA gene region, which is also the barcode region of *Blastocystis* was amplified with a second nested PCR. The PCR reaction was carried out by using the forward BsRD5F (5'-ATCTGGTTGATCCTGCCAGT-3') and reverse BhRDr9R (5'-GAGCTTTTTAACTGCAACAACG-3') barcoding primers (Scicluna et al., 2006). The PCR conditions consisted of initial denaturation for 3 min at 94 °C, 35 cycles at 94 °C for 1 min, annealing 60 °C for 1 min, and extension at 72 °C for 100 sec, with a final elongation step at 72 °C for 10 min. Positive and negative controls were included with each batch of samples analyzed.

5.2.8 Quantitative Polymerase Chain Reaction (qPCR)

Blastocystis prevalence was assessed using qPCR to amplify a 330 bp fragment of the *SSU* rRNA gene. The qPCR reactions mixture were performed in 10 µl reaction mixture volume with 3 µl of water, 4 µl SensiFASTTM SYBR No-ROX Kit (BIOLINE, UK), 0.5 µl of each forward (BL18SPPF1; 5'-AGTAGTCATACGC TCGTCTCAAA-3') and reverse (BL18SR2PP; 5'-TCTTCGTTACCCGTTACTGC-3') *Blastocystis*-specific primer and 2 µl of genomic DNA. The qPCR amplification conditions were as previously described (Poirier et al., 2011). Reactions were run in 96-well plates in a CFX96 Touch™ Real-Time PCR Detection System (Bio-Rad, USA). Positive and negative controls were used in each qPCR run together with all samples. Each type of sample was run separately to avoid cross-contamination. For example, soil sample experiments were executed on separate plates and on separate days from water, human and animal samples.

5.2.9 Amplicon Purification and Sequencing

All positive PCR and qPCR products were purified using the GeneJET Gel Extraction Kit (Thermo Scientific; Wardmedic, Thailand) according to manufacturer's instructions and sequenced at U2Bio (Korea).

5.2.10 Cloning

Twenty-one samples showing long stretches of indistinguishable peaks were cloned, six of which were PCR products and 15 qPCR. Five samples were human, two buffalo, two pig, four chickens, four water and four soil. 1.5 μL of amplicon was used with the pGEM-T easy vector system I (Promega, Madison, WI, USA) following previously published cloning protocols (Betts et al., 2018). Up to five colonies per transformation were screened.

5.2.11 Phylogenetic Analysis

The chromatogram quality of raw reads was checked using the chromatogram visualization software 4Peaks. Ambiguous bases at the ends of the reads were removed. The new sequences were then used as queries to perform blast searches against the NCBI nr database. Sequences of *SSU* rRNA spanning the spectrum of *Blastocystis* diversity were downloaded and aligned using mafft v. 7.394 (Katoh & Toh, 2010). Ambiguous positions were removed using trimal v. 1.4 and gappyout option (Capella-Gutierez et al., 2009). The final trimmed alignment consisted of 250 taxa and 1497 sites. Maximum likelihood (ML) analysis was performed in CIPRES Science Gateway (n.d.), (Miller et al., 2010) using RAxML-HPC2 on XSEDE. Bootstrap support was computed from 1000 pseudoreplicates.

5.3 Results

5.3.1 Human Demographic Data

A total of 45 human volunteers participated in this study (31 % male, n=14 and 69% female, n=31), with mean age of 59.1 ± 8.5 years (median=60).

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5.3.2 Comparison of Microscopy and Molecular Methods in Human Stool Samples

The prevalence of *Blastocystis* in all human stool samples was observed using morphology and molecular techniques (Table 5.1). All samples were cultured in LYSGM and of these, 9% (4/45) were microscopy-positive for *Blastocystis*. Using conventional PCR, 49% (22/45) of samples were positive, while the number increased to 73% (33/45), when using qPCR. All microscopy-positive samples were also positive using molecular detection. Eleven PCR samples were false positive by Sanger sequencing (plants and fungi rather than *Blastocystis*), thus PCR positivity rate of *Blastocystis* confirmed by sequencing was 27% (12/45). One qPCR product was false positive by Sanger sequencing (Fungi; not included in the prevalence calculation). The prevalence rates reported are based only on samples that have been sequenced and are indeed verified as *Blastocystis*.

Table 5.1 Comparison of microscopy and molecular methods

Methods	Prevalence	
	Positive	**Negative**
Morphology		
Light microscopy	4 (8.89%)	41 (91.11%)
Molecular		
Polymerase chain reaction (PCR)	12 (26.67%)	33 (73.33%)
quantitative Polymerase chain reaction (qPCR)	34 (75.56%)	11 (24.24%)

5.3.3 Prevalence and Diversity of *Blastocystis* in Animal and Environmental Samples

Forty-four faecal samples were collected from animals as follows: chickens (n=34), pigs (n=6) and buffalo (n=4). All animal samples were cultured in LYSGM. Using microscopy, 65% (22/45) of chicken cultures were positive, while no *Blastocystis* was observed in pig and buffalo cultures. Using qPCR and subsequent sequencing, the prevalence of *Blastocystis* was 100% in chickens, pigs and buffalo.

Overall, 28 samples of water and seven samples of soil were cultured and surveyed for *Blastocystis*. Two water and one soil sample were false positives for Cercozoa and bacteria and were not considered for further analysis. Prevalence using qPCR was 93% (26/28) for water and 86% (6/7) for soil. The reported prevalence rates are based solely on samples that have been sequenced and verified as *Blastocystis*.

Of the PCR and qPCR *Blastocystis* positive samples that were sequenced, 21 were cloned: Cloning yielded 62 clones, of which 17 were from human fecal samples, six from buffalo, nine from pig, 14 from chicken, nine from water and seven from soil (Supplementary material 2). The following subtypes (STs) were identified: ST1, ST2, ST3, ST4, ST5, ST6, ST7, ST10, ST23 and ST26 (Table 5.2). Nine sequences could not be subtyped either because of poor quality or short length. Eight of the identified subtypes were found in humans. The dominant subtype was ST23 (12/33, 36%), followed by ST10 (6/33, 18%), ST1 (5/33, 15%), ST3 (2/33, 6%) and a single occurrence of ST2 (1/33, 3%), ST4 (1/33, 3%), ST5 (1/33, 3%) and ST7 (1/33, 3%). Chickens carried ST6 (2/33, 3%) and ST7 (31/33, 94%), pigs ST7 (6/6, 100%) and buffalo ST7 (2/4, 50%) and ST23 (2/4, 100%). Subtype 1 (5/26, 19%), ST3 (13/26, 50%), ST6 (1/26, 4%), ST7 (1/26, 4%), ST23 (1/26, 4%) and ST26 (2/26, 8%) were detected in water, whereas in the soil samples ST1 (1/6, 17%), ST3 (2/6, 33%), ST7 (2/6, 33%), ST23 (3/6, 50%) and ST26 (2/6, 33%) were found. Three humans carried both ST10 and ST23. Within subtypes, multiple genetically diverse strains were present in ST7, while ST1, ST3, ST5 and ST6 sequences were much more genetically similar (data not shown).

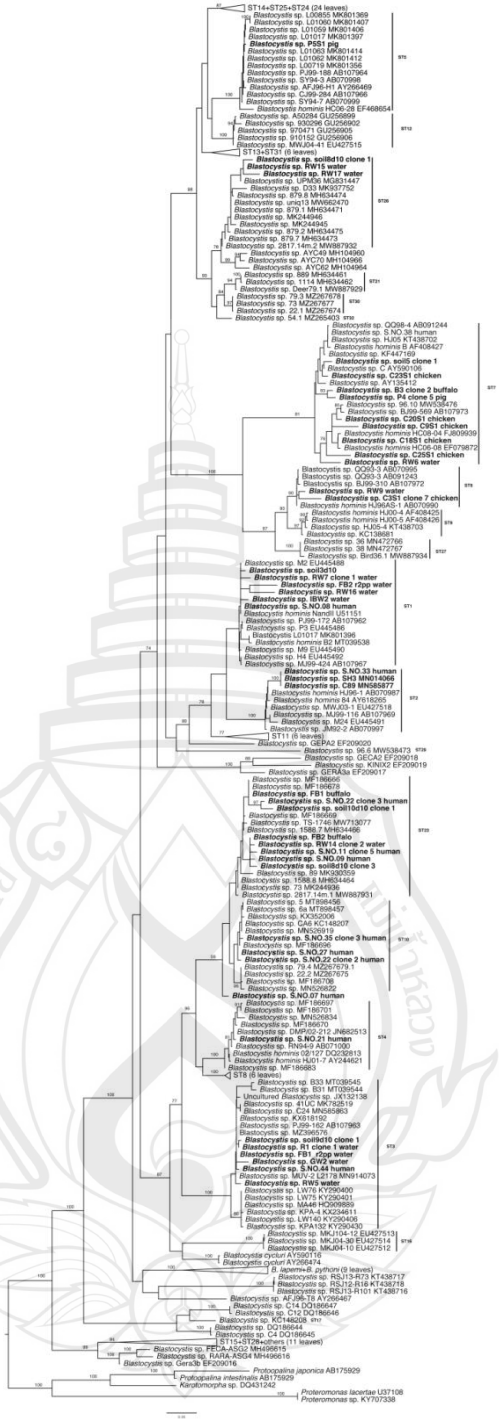
A detailed account of all newly generated sequences is provided in Supplementary Material 2. All 149 sequences generated in this study have been submitted to GenBank under accession numbers OL351649 - OL351797.

Table 5.2 Prevalence and subtypes of *Blastocystis* in human, animal, water and soil samples

Source	B +ve	ST1	ST2	ST3	ST4	ST5	ST6	ST7	ST10	ST23	ST26	UNK
Human	33	5	1	2	1	1	-	1	6	12	-	1
Chicken	33	-	-	-	-	-	2	31	-	-	-	-
Pig	6	-	-	-	-	-	-	6	-	-	-	-
Buffalo	4	-	-	-	-	-	-	2	-	2	-	-
Water	26	5	-	13	-	-	1	1	1	1	2	2
Soil	6	1		2	-	-	-	2	-	3	1	-
Total	**108**	**11**	**1**	**17**	**1**	**7**	**3**	**37**	**7**	**18**	**3**	**3**

5.3.4 Phylogenetic Analysis

All *Blastocystis* sequences grouped together with maximum bootstrap support (BS) (Figure 5.3). Subtypes 15 and 28 along with sequences from ectothermic hosts placed in the base of the tree in agreement with previous studies (Higuera et al., 2021). Subtype 5, ST12, ST13, ST14, ST24 and ST25 formed a clade sister to the clade formed by ST26, ST21, ST30 and ST32. Distinct clades of subtypes were as follows: ST6 and ST7; ST1, ST2 and ST11; ST23 and ST10; and ST4 and ST8. Newly generated sequences placed within clades consisting of known subtypes with the exception of the human origin sequence S.NO.07. Notably, the positions of the new sequences placing with ST10 and ST23 are not entirely robust, suggesting that perhaps these are new, closely related subtypes. Nonetheless, without full length sequences further conclusions cannot be drawn (this is currently under investigation).



Note The tree is artificially rooted to *Proteromonas*, *Protoopalina* and *Karotomorpha* sequences. Newly generated sequences are in bold. Numerical values indicate bootstrap support. Only values above 70% are depicted

Figure 5.3 Maximum likelihood phylogenetic tree inferred from 250 taxa and 1497 sites of the *SSU* rRNA gene

5.3.5 Transmission Dynamics

Household level. Samples were collected from a total of 39 households. In most cases, a single individual per household was sampled, with the exception of six households where all samples from all individuals were collected (Figure 5.1). Of those, five households were found positive for *Blastocystis.* In two of them, only the male occupant was positive. In the other three households, both occupants were positive, but carried different subtypes.

Farm animal ownership level. Of the 39 sampled households, eight of them had animals (seven with chickens and one with buffalo). *Blastocystis* was found in six of these households and there was no subtype sharing between animal and human hosts (Table 5.3).

Table 5.3 Prevalence of *Blastocystis* in animals and their animal-keepers

Household	**Animals**	***Blastocystis* in humans**	***Blastocystis* in animals**
1	chicken	Negative (n=2)	ST7 (n=10)
2	chicken	ST1 (n=1) ST10 (n=1)	ST7 (n=5)
3	chicken	ST23 (n-1) unknown (n=1)	ST7 (n=5)
4	chicken	ST23 (n=1)	ST7 (n=3)
5	chicken	ST3 (n=1)	ST7 (n=4)
6	chicken	Unknown (n=1)	ST7 (n=3)
7	buffalo	ST4 (n=1)	ST23, ST7 (n=4)
8	chicken	Negative (n=1)	ST7 (n=4)

Environmental level. Of the 39 sampled households, 16 were sampled for water and six for soil, all of which were positive for *Blastocystis*. There was subtype overlap between water and humans in one household (ST3).

Community level. Out of the 108 *Blastocystis* positive samples, 33 (31%) were from humans, 43 (40%) from animals, 26 (24%) from water and 6 (6%) from soil. Subtype 2 and ST4 were identified only in humans, whereas ST26 was only found in the environmental samples (both soil and water). Subtype 7 was the most broadly distributed as it was found in humans, pig, buffalo and chicken, but also in soil and water. Subtype 1, ST3, ST7, ST10 and ST23 were found in human and environmental samples. No subtype was exclusively shared by only humans and other animals. Subtype 6 was the only one shared between animals and the environment.

5.4 Discussion

The study took place in a century old isolated rural community in northern Thailand comprising approximately 500 people. Inhabitants live in very close proximity to their animals primarily chickens and secondarily buffalo and pigs. Part of the community's water supply comes from the river that runs through it. The river also provides a major food source for the villagers, as fish constitutes the primary protein source of the community, along with vegetables (which also grow inside the river and the river bank) and locally farmed sticky rice. The increased influence of westernized diet noted in urban centers of Thailand has a minor impact in this community. Collectively, the small population, distance from urban centers, unique gastronomy (minimal effect from westernization) and the general lifestyle make this particular community ideal for local One Health approaches. Herein, we used *Blastocystis*, a microbial eukaryote of controversial pathogenicity, to obtain a comprehensive view of its transmission dynamics.

Blastocystis is the most frequently encountered intestinal protist of metazoans with most studies focusing on either its prevalence in humans, other animals and/or the environment. Nonetheless, only very few investigations explore the organism's transmission dynamics using a tripartite approach, whereby all of the aforemention

factors are considered collectively. In order to understand the role of this organism in health and disease it is essential to determine its occurrence simultaneously in human and non-human hosts and environments.

In humans, the prevalence of *Blastocystis* has been frequently reported in those with and without gastrointestinal symptoms (Dogruman-Al, Yoshikana et al., 2009b; Scanlan et al., 2014; Lhotská et al., 2020; Padukone et al., 2021; Yowang et al 2018; Kataki, Tavalla & Beiromvand, 2019). Overall prevalence of *Blastocystis* might vary due to sampling population, region and detection method (Stensvold et al., 2009b; Tan et al., 2010; Alfellani, Stensvold et al., 2013; Anuar et al., 2013; Clark et al., 2013). Herein, the prevalence of *Blastocystis* in asymptomatic human hosts was 73%, in asymptomatic non-human hosts 100% and in environmental samples also 91%. We used microscopy and molecular methods to determine presence of *Blastocystis*. The most sensitive detection method was qPCR matching previous studies (Poirier et al., 2011; Stensvold et al., 2012). After sequencing all positive samples, a broad diversity of subtypes (STs) was detected: ST1, ST2, ST3, ST4, ST5, ST6, ST7, ST10, ST23 and a potential new subtype. Subtype 10 was detected in six human volunteers. The subtype has been previously found in two Senegalese children (Khaled et al., 2020), but it is a typical cattle subtype (Cian et al., 2017, Zhu et al., 2017, Masuda et al., 2018; Wang et al., 2018). To our great surprise, we found ST23 in 12 human samples making it the dominant subtype in this host. So far, ST23 has only been identified in ruminants. The occurrence of ST10 and ST23 in several adults in an Asian country raises questions regarding the host range and transmission dynamics of *Blastocystis* subtypes.

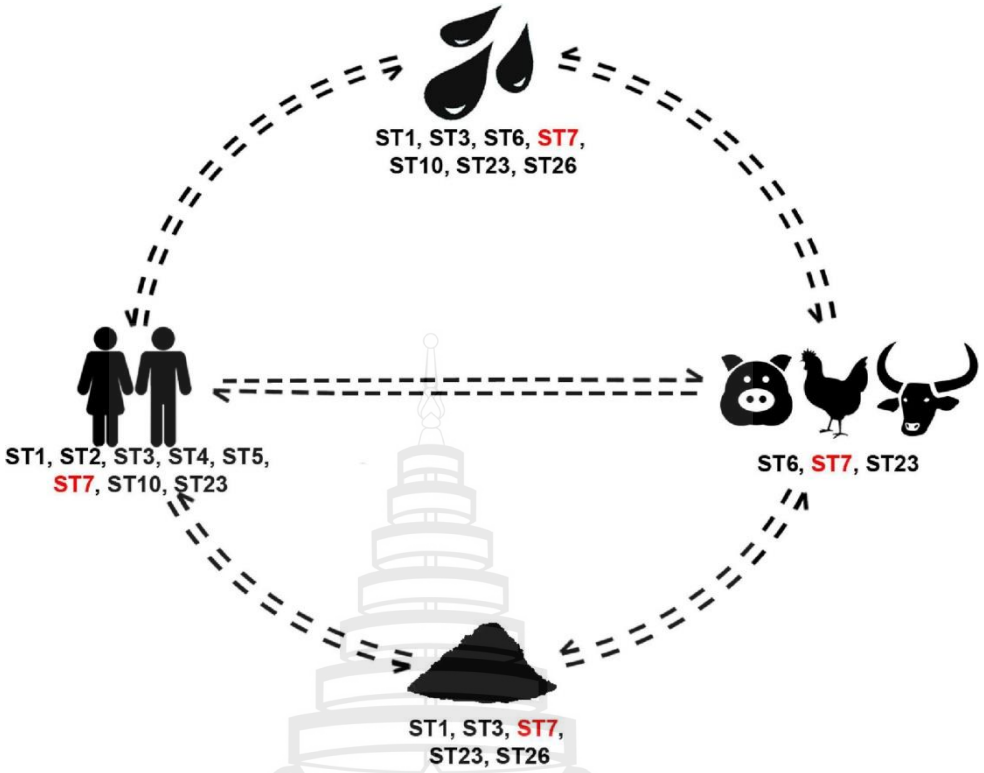
The following transmission routes have been widely discussed for *Blastocystis*: human-to-human, animal-to-human and environment-to-human. The former mode of transmission has been speculated to occur via the faecal-oral route much like other common gastrointestinal parasites. Herein, investigation of individuals within households showed no subtype sharing and there was even an instance of co-habiting individuals, whereby one was *Blastocystis* positive and another negative. This finding matches previous recent reports derived from family units elsewhere (Scanlan et al., 2016; Lhotska et al., 2020).

We also aimed to look at the animal-to-human transmission route. Previous studies have suggested that specific subtypes are zoonotic (Alfellani, Taner-Mulla et al., 2013; Parkar et al., 2010; Wang et al., 2014). For instance, ST5 has been proposed as potentially zoonotic from pigs (Yan et al., 2007; Wang et al., 2014) and *Blastocystis* ST6 and ST7 from poultry (Ramirez et al., 2014; Cian et al., 2017; Greige et al., 2018; Udonsom et al., 2018). Subtype 1, ST7, ST10 and ST23 were found in both human and animal hosts in the studied area giving the impression of zoonotic transmission. However, when looking at a fine-scale level there was no sharing of subtypes between animals and their respective owners. Collective consideration of the evidence points towards the source of *Blastocystis* in this specific community being elsewhere.

This prompted us to look at the two most commonly encountered environmental sources in the community: water and soil. Water contamination has been speculated as a risk factor to acquire *Blastocystis*. However, only few studies have looked at presence of *Blastocystis* in both water and humans that use it and even fewer have employed subtyping to examine overlap between the two (Leelayoova et al., 2008; Angelici et al., 2018; Pawestri et al., 2021). *Blastocystis* has been detected in drinking water (Leelayoova et al., 2008), tap water (Eroglu & Koltas, 2010), rain water tanks (Noradilah, Moktar et al., 2017b; Waters et al., 2019), bodies of freshwater (Ithoi et al., 2011; Khalifa et al., 2014), drinking water treatment facilities (Richard et al., 2016) and waste water (Suresh et al., 2005; Banaticla & Rivera, 2011, Stensvold et al., 2020). Herein, *Blastocystis* ST1 and ST3 were detected in community supply water, while ST1, ST3, ST5, ST6, ST7, ST10, ST23 and ST26 were found in rain collection vessels. Both these sources comprise the drinking water of this community. The rain collection vessels contain water that is filtered for large debris, but the water is untreated and is consumed unboiled (Li et al., 2007; Leelayoova et al., 2008; Anuar et al., 2013; Wongthamarin et al., 2018; Waters et al., 2019). The community supply water is filtered and occasionally treated. Given the exposed nature of the community water, various wildlife animal hosts harboring a range of subtypes (known and unknown) can easily access it. Thus, presence of the organism in these two sources could be due to a combination of factors including

contamination by animal droppings and/or substandard management (i.e. filtration and chlorine usage). Water in the vessels is also used to wash vegetables and tubers hence transfer of cysts of a variety of subtypes could occur this way. Indeed *Blastocystis* has been previously found in vegetables (Al Nahhas & Aboualchamat, 2020; Li et al., 2020). Through fine-scale analysis we identified a case of ST3 in humans overlapping with the subtypes found in their rain collection vessels. Presence of *Blastocystis* in an environment, where there is continuous circulation of oxygen supported recently raised hypotheses that this previously considered strictly anaerobic organism tolerates oxygen (Tsaousis et al., 2012; Tsaousis et al., 2018). Thus, future studies should aim towards investigating additional environments including extreme habitats for the presence of *Blastocystis*.

To that end, we broadened our approach and also explored occurrence of *Blastocystis* in soil. Most collected soil samples were positive for the organism, while ST1, ST3, ST7, ST23 and ST26 were identified. To our knowledge this is the first report of this protist being recorded in natural soil. The presence of *Blastocystis* in the soil could be due to extensive use of animal excrement and intestinal contents (especially from fish), which are typically utilized as garden fertilizer in the community. Nonetheless, while sampling, care was taken to collect from gardens that had not been recently fertilized. Moreover, wildlife hosts roaming the community could also shed *Blastocystis*. This finding suggests a new route of transmission that has been previously overlooked. In that vein, we propose that soil should not only be checked for presence of the organism in future studies, but that it should also be included along with water as a transmission route in the life cycle of *Blastocystis* (Figure 5.4).



Note Subtypes present in all sources are in red font

Figure 5.4 *Blastocystis* subtype cycling in the rural community studied herein

Comparison of sequences found in different hosts and environments indicated that highly similar strains of ST1 and ST3 are circulating in the community. This suggests a shared *Blastocystis* transmission cycle among humans, animals and the environment for these subtypes. In contrast, ST7 showed an extraordinary amount of diversity with multiple strains distributed within and between hosts and the environment. This indicates that the full extent of ST7 genetic diversity and host range in the community has yet to be captured. Nonetheless a cluster of highly similar strains was found in chicken, pig and buffalo suggesting transmission among the three hosts.

This study has revealed no clear patterns of direct transmission between human-to-human or animal-to-human in this community (Figure 4). Instead, it points out to the existence of multiple independent routes of transmission. Previous efforts investigating *Blastocystis* sources of transmission have been geared towards dissecting dipartite relationships (i.e. animal-to-human or environment-to-human).

Results from these studies have enhanced our understanding of the organism and its epidemiology. Nonetheless, they frequently only provide pieces of the overall picture, which remains fragmentary. Here, we have provided a step forward towards integrating a One Health approach to *Blastocystis* by considering both living and non-living sources. In this community, environmental sources comprise the reservoir of *Blastocystis* supplying a multitude of subtypes that circulate in both human and non-human hosts. Our study is pioneer in that we investigated a rural area, while taking into account the community structure and environmental factors towards understanding *Blastocystis* circulation.

Limited sample size does pose a limitation in our study. Specifically, the sample size was low, in particular samples from various animal hosts including stray animals and wildlife. Thus transmission cycles between and within hosts and the environment cannot be precisely deduced at this time.

Moving forward, additional communities both rural and urban should be explored under the One Health umbrella to determine whether similar patterns occur. Using the same approach in a temporal context, future studies should also investigate, whether *Blastocystis* and its various subtypes are true colonizers or passengers. Finally, supplementing One Health-based studies with culturomics and microbiome (pathogenic and non-pathogenic residents of the gut) and metabolome investigations will contribute significantly in uncovering the true roles of *Blastocystis* in gut health and disease.

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CHAPTER 6

BLASTOCYSTIS IN TAP WATER OF A COMMUNITY IN NORTHERN THAILAND[4]

Abstract

Blastocystis is the most common protist in the gut of humans and other animals with global distribution. Occasional, this organism has also been reported in the environment. Transmission to humans occurs via the fecal-oral route, while water also comprises an important transmission route. *Blastocystis* has been commonly found in rivers, lakes, and wells. Nonetheless, there is limited data about the prevalence and genetic diversity of *Blastocystis* in tap water. The main aim of this study was to examine the presence of *Blastocystis* subtypes in tap water (n=20) in northern Thailand. Molecular characterization using the small subunit ribosomal RNA was employed to screen for *Blastocystis* and identify the diversity of subtypes in samples. The overall prevalence was 30% with only one subtype encountered in the tap water. Our results indicate that tap water has a potential role of transmission of this subtype in this community. Further investigations should be increasing samples size and identify *Blastocystis* in humans who use this water.

Keywords: *Blastocystis*, Small Subunit Ribosomal RNA, Subtype, Tap Water, Thailand

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6.1 Introduction

Blastocystis is one of the most common protists in the intestinal tract of humans and other animals with worldwide distribution (Beghini et al., 2017; Roberts et al., 2013; Stensvold & van der Giezen, 2018). *Blastocystis* has four morphological forms, namely vacuolar, granular, amoeboid, and cyst (Parija & Jeremiah 2013; Tan, 2008). The advent of molecular methods over the past several years, has revealed that the organism has an astounding genetic diversity. Based on the small subunit ribosomal RNA (SSU rRNA) gene, a total of 28 subtypes (STs) of *Blastocystis* have been identified: ST1-ST17, ST21, ST23-ST32 (Alfellani et al., 2013; Betts et al., 2018; Betts, Gentekaki, & Tsaousis, 2020; Higuera et al., 2021; Maloney et al., 2020; Maloney, da Cunha, et al., 2021; Maloney, Jang, et al., 2021; Stensvold et al., 2009). Subtypes ST1-ST17 have been accepted for a long time, while the rest were proposed more recently. So far, ST1-ST10, ST12, ST14, ST16 and ST23 have been found in humans, with ST1-ST4 being the most frequently reported (Jinatham et al., 2021; Khaled et al., 2020; Osorio-Pulgarin et al., 2021; Stensvold & Clark 2016). Nonetheless, these subtypes, have also been found in other hosts, indicating the non-host specific nature of *Blastocystis*. The exception to this is ST9 which so far has only found in humans.

Blastocystis transmission occurs via the fecal-oral route and it has been proposed that the cyst is the transmissible form (Tanizaki et al., 2005). Several avenues of transmission have been explored including human-to-human and zoonotic, or via contaminated food and water (Ahmed et al., 2018; Ii Lee et al., 2012; Jinatham et al., 2021; Tan, 2008) Zoonotic transmission has been considered as commonly occurring. For example, *Blastocystis* ST5 has been detected in pigs and pig handlers (Pintong et al., 2018; Wang et al., 2014), ST7 has been found in avian hosts and people living within the same area, while ST8 has been reported in human and non-human primates (Alfellani et al., 2013; Greige et al., 2018.; Stensvold et al., 2009). In recent years, water sources have been implicated in the transmission cycle of *Blastocystis*. Presence of the organism has been associated with humans who

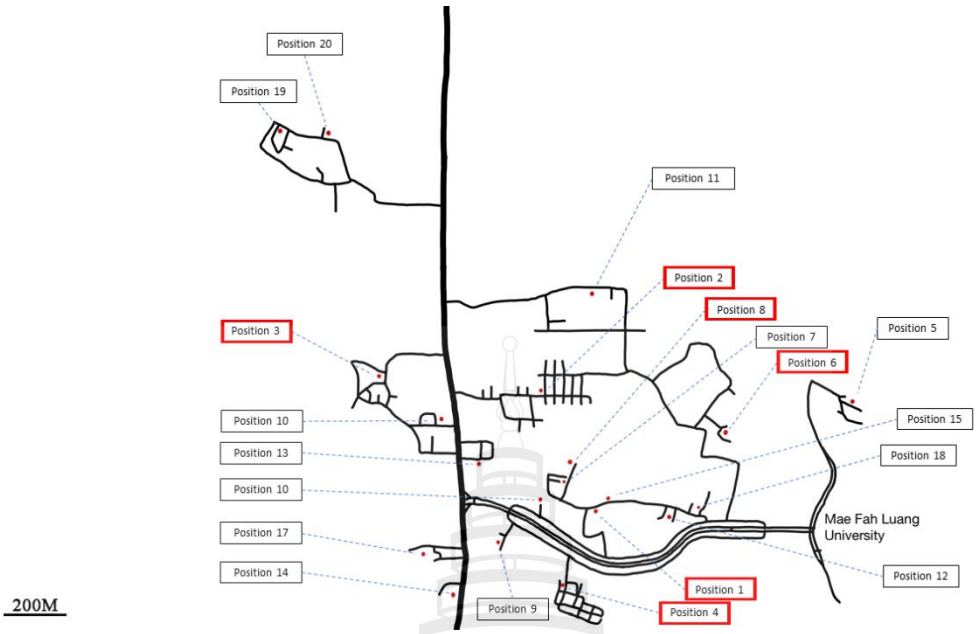
consumed untreated water as well as with those who had no access to piped water supply (Abdulsalam et al., 2012; Taamasri et al., 2000). Several studies have investigated the occurrence of *Blastocystis* in water environments, including rivers, lakes, wells and rain storage containers (Abdulsalam et al., 2012; Adamska, 2020; Banaticla & Rivera 2011; Elshazly et al., 2007; Ithoi et al., 2011; Javanmard et al., 2019; Jinatham et al., 2021; Khalifa et al., 2014; Koloren et al., 2018; Li et al., 2007; Suresh et al., 2005; Taamasri et al., 2000; Waters et al., 2019)

Blastocystis in tap water has been reported only occasionally (Eroglu & Koltas 2010; Leelayoova et al., 2008; Noradilah et al., 2016). In this pilot study we investigated presence of *Blastocystis* in tap water in a semi urban area of northern Thailand. Our objective was to determine whether water comprises a source of acquiring *Blastocystis* in this specific community.

6.2 Methods

6.2.1 Samples Collection, Culturing and Microscopy

Tap water samples were collected from 20 randomly selected housing complexes in Chiang Rai Province, Thailand (Figure 6.1). These complexes house mostly university students. Samples were collected during the wet season between July and August 2021. The source of tap water for 19 housing complexes was verified as coming from groundwater. In 13 of these, water is cleaned by filtration, while in three the tap water is chlorinated. The water in three of the complexes in neither filtered nor chlorinated. The tap water of one of the housing complexes comes from a water reservoir storing river water and is cleaned by filtration (Supplementary figure 6.1A). Tap water was stored in polyvinyl (n=16), stainless steel (n=3), or metal (n=1) containers. Tap water in this study is used for bathing, washing food and clothes and watering plants. Tap water (2-5 ml) was placed in LYSGM as previously described (Eroglu & Koltas, 2010; Jinatham et al. 2021) and incubated at 37 °C. A tube containing only LYSGM media was used as control. All cultures were screened for *Blastocystis* using light microscopy.



Note Red dots indicate housing complexes from which samples were collected. Red squares indicate samples positive for *Blastocysti*

Figure 6.1 Detail of area of collection in this study

6.2.2 Genomic DNA Extraction

Total genomic DNA was extracted using 200 mg of sediment from culture and LYSGM control using high molecular weight extraction protocol (Matsuki et al. 2002). Briefly, 1 mL of each culture was homogenized with cell lysis buffer and incubated at 75 °C. Afterwards, 100 mg/mL of lysozyme was added, and the mixture was incubated at 37 °C overnight. Subsequently, 10% SDS, 25 mg/mL of proteinase K, and 20 mg/mL of RNase were added and incubated at 55 °C for 30 mins. Phenol/chloroform/isoamyl alcohol (25:24:1) was added and the tubes were centrifuged at 13,000 x g for 5 min. Residual contamination of phenol/chloroform was removed by adding chloroform/isoamyl alcohol (24:1) and centrifugation at 13,000 x g for 5 min. The aqueous phase was kept, and the rest discarded. The DNA was precipitated with 3 M sodium-acetate and isopropanol by centrifugation at 13,000 x g for 5 min. The DNA pellet was washed with 70% ethanol, air-dried, and re-suspended in TE buffer.

6.2.3 Quantitative Polymerase Chain Reaction (qPCR)

A fragment of the SSU rRNA gene of *Blastocystis* was amplified using quantitative polymerase chain reaction (qPCR) analysis. This method of detection was chosen due to its sensitivity and low rate of false positives in our previous studies (Jinatham et al. 2021). The SensiFAST™ SYBR No-ROX Mix (Bioline company, U. S. A) was used in the qPCR reaction. The PPF1 (5'-AGTAGTCATACGCTCGTC TCAAA-3) and R2PP (5'-TCTTCGTTACCCGTTACTGC-3') primers were used (Poirier et al., 2011). The expected product size was 330 bp. The qPCR conditions were as follows: initial denaturation at 95 °C for 5 min followed by 49 cycles of denaturation at 95 °C for 5 sec, annealing at 68 °C for 10 sec, and 72 °C for 15 sec. Two negative controls were used; one containing all reagents except template and one containing the DNA extracted from the LYSGM media control. Reactions were run on a Bio-Rad/CFX96 Touch Real-Time PCR Detection System. The amplicon products positive for *Blastocystis* were purified using GeneJET Gel Extraction Kit (Thermo Scientific; Wardmedic, Thailand) according to the manufacturer's instructions and sequenced (Bionics Company, Korea).

6.2.4 Phylogenetic Analysis

The chromatogram quality of all reads was checked using the chromatogram visualization software 4Peaks. Ambiguous bases at the ends of the reads (5' and 3') were removed. The newly obtained sequences were used as queries to perform BLAST searches against the GenBank database to ensure they were not contaminants or chimeras. Subtype Sequence Typing (MLST) (Jolley et al., 2018) was also used to identify subtype and the corresponding allele. The SSU rRNA gene sequences of all currently accepted *Blastocystis* subtypes were used to construct the dataset. In total, 119 sequences were aligned using the software MAFFT v.7 (Katoh & Toh, 2010) and ambiguous regions were removed using trimAL v.1.4 (Capella-Gutiérrez, Silla-Martínez, & Gabaldón 2009). After trimming the alignment contained 1336 sites. A maximum likelihood (ML) phylogenetic tree was built using RAxML v.8 (Stamatakis, 2006) the general time reversible + Γ model of sequence evolution and 1000 bootstrap replicates.

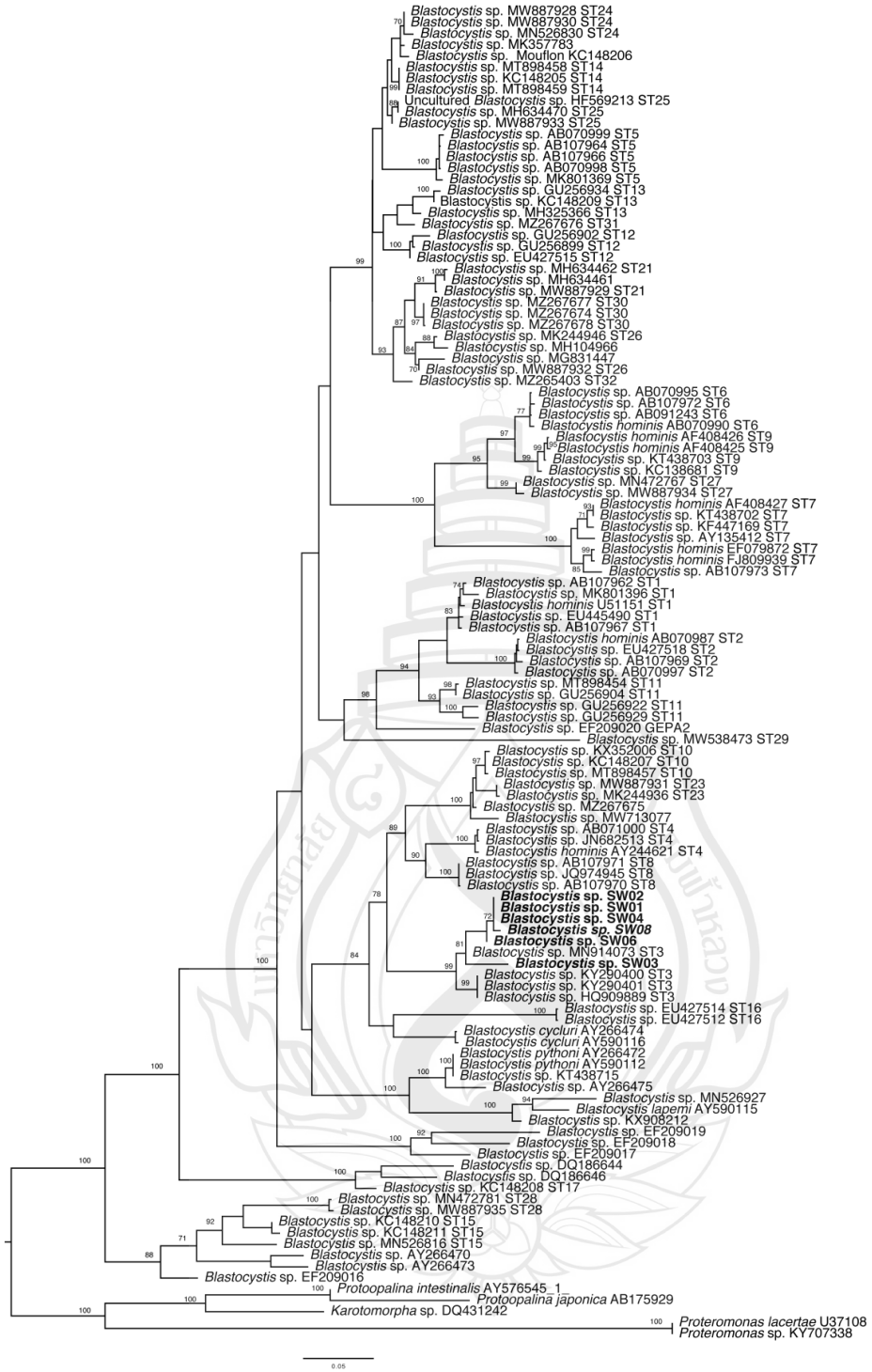
6.3 Results

Sediments from three culture were positive for *Blastocystis* by light-microscopic examination (results not shown). Ten of the 20 tap water samples (50%) were qPCR positive. However, after sequencing, only six of the ten were identified as *Blastocystis*. The other four were falsely positive and represented sequences that cannot be identified in the database. Thus the overall occurrence of *Blastocystis* in tap water was 30%. All six *Blastocystis* positive sequences were identified from tap water coming from groundwater. Of these six, one sample was neither chlorinated nor filtered, three were chlorinated but not filtered, and two were filtered, but not chlorinated (Table 6.1). Water from all six samples was stored in polyvinyl containers after filtration/chlorination.

Table 6.1 Water containers that were sampled in this study and their corresponding purification systems

Position	Container type	Chlorine	Filter	Positive
1	Polyvinyl	-	-	+
2	Polyvinyl	+	-	+
3	Polyvinyl	-	+	+
4	Polyvinyl	+	-	+
5	Metal	-	+	-
6	Polyvinyl	-	+	+
7	Polyvinyl	-	+	-
8	Polyvinyl	+	-	+
9	Polyvinyl	-	+	-
10	Polyvinyl	-	+	-
11	Stainless steel	-	+	-
12	Polyvinyl	-	-	-
13	Polyvinyl	-	+	-
14	Stainless steel	-	+	-
15	Stainless steel	-	+	-
16	Polyvinyl	-	+	-
17	Polyvinyl	-	+	-
18	Polyvinyl	-	-	-
19	Polyvinyl	-	+	-
20	Polyvinyl	-	+	-

In the phylogenetic analysis, all six newly derived sequences claded with ST3 (Figure 6.2). However, the possibility of other subtypes being present in the water and not established in culture cannot be excluded. All subtypes were monophyletic. In agreement with previous studies, *Blastocystis* isolates from ectothermic hosts along with ST15, and ST28 placed at a basal position. The closest allele match for all six sequences was allele 34. Sequence SW06 was identical to that of allele 34 in the MLST database, sequences SW01, SW02 and SW04 differed by a single nucleotide, while sequences SW08 and SW03 had two and five differences, respectively.



Note Newly generated sequences are depicted in bold font. Numerical values at the nodes indicate bootstrap support. Values below 70% are not shown

Figure 6.2 Maximum likelihood phylogenetic tree inferred from 1336 sites of the SSU rRNA gene and 119 taxa

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6.4 Discussion

In this pilot study we surveyed housing complexes for the presence of *Blastocystis* in tap water following previously established protocols (Eroglu & Koltas, 2010). Presence of *Blastocystis* in tap water has not been frequently explored, and in Thailand such surveys are sparse. *Blastocystis* was identified in 30% of the sampled tap water.

Though *Blastocystis* has been reported in a variety of water environments, including treated potable rain water, water tanks, ponds, canal water and wastewater, subtype information is not always available (Elshazly et al., 2007; Khalifa et al., 2014; Suresh et al., 2005; Waters et al., 2019). Nonetheless, a variety of subtypes have been identified in water. Subtype 1 has been reported in drinking water, non-potable water and tap water (Eroglu & Koltas, 2010; Leelayoova et al. 2008), while ST1, ST2, ST6 and ST8 have been found in wastewater treatment plants (Banaticla & Rivera, 2011; Javanmard et al., 2019). A variety of *Blastocystis* subtypes (ST1, ST3, ST6, ST7, ST10, ST23 and ST26) were found in water bodies and containers in a rural community in northern Thailand (Jinatham et al., 2021). Worldwide, ST1-4 have been identified in river samples (Adamska, 2020; Ithoi et al., 2011; Koloren et al., 2018; Noradilah et al., 2016). The organism has also been found in unboiled drinking water, rivers, tap water as well as natural water bodies and rain water collection tanks (Abdulsalam et al., 2012; Adamska, 2020; Banaticla & Rivera, 2011; Elshazly et al., 2007; Eroglu & Koltas, 2010; Ithoi et al., 2011; Javanmard et al., 2019; Jinatham et al., 2021; Khalifa et al., 2014; Koloren et al., 2018; Leelayoova et al., 2008; Li et al., 2007; Noradilah et al., 2016; Suresh et al., 2005; Taamasri et al., 2000; Waters et al., 2019). Subtype 1 and ST3 are the most commonly encountered in water sources, while ST2, ST4, ST6-8, ST10, ST23 and ST26 have occurred only sporadically (Adamska, 2020; Banaticla & Rivera 2011; Eroglu & Koltas 2010; Ithoi et al., 2011; Javanmard et al., 2019; Jinatham et al., 2021; Koloren et al., 2018; Leelayoova et al., 2008; Li et al., 2007; Noradilah et al., 2016). Herein, all samples positive for *Blastocystis* belonged to ST3. No other subtypes were detected. This is in contrast to the study by Jinatham et al. (2021), who detected several subtypes in water.

One explanation for this could be that the media used might encourage growth of some subtypes, but not others. Both studies used the same media (LYSGM) so one can at least conclude that several subtypes can grow in this medium. Our study suggests *Blastocystis* ST3, is circulating in the water of the sampled community and poses a possible risk factor of *Blastocystis* transmission. Thus, more studies examining occurrence of *Blastocystis* from various bodies of water and geographical regions are needed to understand distribution of this organism in the environment.

Contaminated water has been implicated as a source of transmission for this organism (Jinatham et al., 2021; Leelayoova et al., 2008; Noradilah et al., 2016). Nonetheless, only a few studies have demonstrated that water is a route of *Blastocystis* transmission by examining both water and human hosts. For instance, *Blastocystis* ST1 and ST3 were positively associated with untreated water and drinking unboiled water, respectively (Leelayoova et al., 2008; Li et al., 2007). Jinatham et al. (2021) identified *Blastocystis* ST3 in humans and in water from their rain collection vessels. Previous studies have shown high prevalence of *Blastocystis* ST1 and ST3 in people who consumed untreated or contaminated water (Eroglu & Koltas, 2010; Li et al., 2007).

To our knowledge, not many studies have looked for *Blastocystis* in tap water directly. The findings herein match those of Eroglu and Koltas (2010) in Turkey, who also found *Blastocystis* in tap water. Leelayoova et al. (2008) reported *Blastocystis* ST1 in drinking water consumed by elementary school children in a rural Thai community. In this study water in which *Blastocystis* was identified was either chlorinated or filtered, but not both. The presence of *Blastocystis* in tap water that is filtered was surprising. One possibility is that the organism is present in the groundwater supply. Animal feces contaminated with *Blastocystis* might be transferred into the groundwater with soil and/or sand. Alternatively, *Blastocystis* could have entered the storage container after filtration since water can sit in the container for several days. A potential alternative possibility is that the organism cannot only escape conventional water filtration, but is also resistant to chlorine. The frequency of filter changing and chlorination standards for each residence are

unknown. *Blastocystis* resistance to chlorine had been previously explored (Leelayoova et al. 2008; Zaki et al., 1996). Moreover, it has been proposed that *Blastocystis* can also survive in water for long periods of time (Moe et al., 1996).

The present study has several limitations including a small sample size. Future studies should increase sample size in terms of both number of housing complexes and residences within the complexes. Samples should also be taken from the storage containers directly as well as the groundwater and compared with those found in the taps. Finally, it would be interesting to see whether the human occupants of the *Blastocystis* positive dorms are also positive for the organism. Our study is one of the few that explore *Blastocystis* occurrence in tap water. Further investigations under the umbrella of one health should be undertaken in the future to understand the distribution of the organism in the environment and the water supply and consequently its transmission dynamics.

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CHAPTER 7

CONCLUSION

This study examined the diversity of eukaryotic microbiome in the gut of animals and humans from rural and suburban areas.

Herein, the diversity of microbial eukaryotes was studied in gut of animals that are widely and commonly found in rural and suburban areas. *Entamoeba chiangraiensis* is a novel species present in the gut of Asian swamp eel (*Monopterus albus*), which inhabits rivers across Southeast Asia. Detailed morphological description and molecular data were determined. This is the first molecular data of an *Entamoeba* species from fish helps fill previous gap knowledge in ectotherms. *Blastocystis* has been found in various hosts such as pig, chicken, and buffalo, but there are some notable differences in subtype. In this study ST5, ST7, and ST10 were found in pig, chicken, and buffalo, respectively. This study adds on to the knowledge of *Blastocystis* subtype distribution in different hosts in Thailand.

Additionally, this study has identified microbial eukaryotes in people 86 living in rural and suburban areas. *Prototheca* is a green alga and two most common species causing the human infection are *P. bovis* and *P. wickerhamii*. Herein *Prototheca* was identified in the stool of four individuals that exhibited no diarrhea living in rural and sub-urban. Most of the people in the area were agriculturists and have frequent contact with ruminants and poultry, whose dung is used to fertilize their vegetable gardens. All four *Prototheca* isolates were characterized by microscopic examination and for the first time molecularly by using the *SSU* rRNA and *cytb* genes. The isolates were identified as *P. bovis*. Cells were found in the stool in various cell division stages, which suggests that the organism remained in the host long enough to undergo cell division. Hence, this study reports for the first time of *Prototheca* species in gut of asymptomatic person but one cannot exclude the possibility of P. *bovis* asymptomatically colonizing the hosts.

Here we also found *Blastocystis* in the gut of humans living in rural areas. Diversity and prevalence of *Blastocystis* in this study were identified using both morphological and molecular approaches. Prevalence rates of *Blastocystis* were 73% in human hosts (n = 45) and the subtypes identified were ST1, ST2, ST3, ST4, ST5, ST7, ST10, and ST23. This study provides the first insight into the *Blastocystis* in people living in rural areas and we are the first to report *Blastocystis* ST10 in Thailand. To great surprise, this is the first globally report *Blastocystis* ST23 in a human host. The occurrence of ST10 and ST23 have only been identified in ruminants.

Transmission mode has been widely discussed for *Blastocystis*: human-to-human, animal-to-human, and environment-to-human. Herein, investigation of individuals within households showed no subtype sharing and there was even an instance of co-habiting individuals, whereby one was *Blastocystis* positive and another negative. This finding matches previous recent reports derived from family units elsewhere. In order to understand the role of zoonosis we observed the animals such as chickens, and buffalo from the six households. We found positive *Blastocystis* in humans and their animals but different subtypes. These findings could help to ascertain the debate on the transmission of *Blastocystis* between animals and their respective owners is minimal. Moreover, we investigated *Blastocystis* in commonly encountered environmental sources in the community, including water and soil. The prevalence of *Blastocystis* has been found to overlap subtypes between the water source and owner house. Collective consideration of the evidence points toward waterborne transmission of *Blastocystis* to humans. To that end, we observed more water sources and we found 30% *Blastocystis* in tap water which subtype 3. This was showed that *Blastocystis* can survive in water and also, supported water transmission mold. The explored occurrence of *Blastocystis* in soil and we found most collected soil samples were positive for the organism which ST1, ST3, ST7, ST23, and ST26. This finding suggests a new route of transmission that has been previously overlooked. Interestingly, this result is the first present of one health approach on *Blastocystis i*n a rural community in Thailand. In future studies should aim toward investigating additional environments including extreme habitats for the presence of *Blastocystis*.

Future directions, diversity and prevalence of microbial eukaryotes should be explored under the One Health umbrella for examination in additional communities both rural and urban in the world. The supplementing One Health-based studies with culturomics and microbiome (pathogenic and non-pathogenic residents of the gut) and metabolome investigations will contribute significantly to uncovering the true roles of microbial eukaryotes in gut health and disease. Finally, investigation of microbial eukaryotes are true colonizers or passengers in gut human and animals.

APPENDIX

APPENDIX

Parasitology

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Entamoeba chiangraiensis n. sp. (Amoebozoa: Entamoebidae) isolated from the gut of Asian swamp eel (*Monopterus albus*) in northern Thailand

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Abstract

The genus *Entamoeba* comprises mostly gut parasites and commensals of invertebrate and vertebrate animals including humans. Herein, we report a new species of *Entamoeba* isolated from the gut of Asian swamp eels (*Monopterus albus*) in northern Thailand. Morphologically, the trophozoite is elongated and has a single prominent pseudopodium with no clear uroid. The trophozoite is actively motile, 30–50 μm in length and 9–13 μm in width. Observed cysts were uninucleate, ranging in size from 10 to 17.5 μm in diameter. Chromatin forms a fine, even lining along the inner nuclear membrane. Fine radial spokes join the karyosome to peripheral chromatin. Size, host and nucleus morphology set our organism apart from other members of the genus reported from fish. The SSU rRNA gene sequences of the new isolates are the first molecular data of an *Entamoeba* species from fish. Phylogenetic analysis places the new organism as sister to *Entamoeba invadens*. Based on the distinct morphology and SSU rRNA gene sequence we describe it as a new species, *Entamoeba chiangraiensis*.

Introduction

Entamoeba is a member of the Entamoebidae, a deep lineage within the Archamoebae (Pánek *et al.*, 2016). *Entamoeba* species use pseudopodia for locomotion and lack flagella, a morphologically identifiable Golgi apparatus, peroxisomes and canonical mitochondria (Loftus *et al.*, 2005; Ptáčková *et al.*, 2013). *Entamoeba* species have trophozoite and cyst stages. The latter may have one nucleus or as many as eight, each with peripheral chromatin prominently visible. Historically, cyst size and nuclear number and appearance, along with host range information, were considered taxonomically important features and used to identify and group species of *Entamoeba*. However, in recent years it has become obvious that morphological features alone are not sufficient to adequately discriminate species known to be genetically distinct (Clark *et al.*, 2006; Stensvold *et al.*, 2011). For example, morphology does not distinguish the morphologically identical *E. histolytica* and *E. dispar*, yet only the former is a human pathogen (Gonin and Trudel, 2003; Fotedar *et al.*, 2007*a*; Hooshyar *et al.*, 2015). The advent of molecular tools has shed light on the taxonomic landscape of *Entamoeba* and clarified several issues associated not only with taxonomy but also epidemiology and host range (Verweij *et al.*, 2003; Fotedar *et al.*, 2007*b*; García *et al.*, 2014). Screening of fecal samples from a broad range of hosts using SSU rRNA gene primers has uncovered several new and distinct lineages of *Entamoeba*, indicating a richly diverse genus (Santos *et al.*, 2010; Stensvold *et al.*, 2011; Jacob *et al.*, 2015). Much of this diversity had not been previously recognized.

Members of the genus *Entamoeba* generally inhabit the gastrointestinal tract of vertebrates and invertebrates, but they have also been observed within other protist cells (Ghosh, 1973; Stensvold *et al.*, 2011; García *et al.*, 2014; Shilton *et al.*, 2018). Several *Entamoeba* species are parasitic, but commensals are more common (Hooshyar *et al.*, 2015). Uniquely among members of the genus, *E. gingivalis* inhabits the human oral cavity (Ghabanchi *et al.*, 2010; Luszczak *et al.*, 2016; Maybodi *et al.*, 2016). In addition, a few members of the genus have also been isolated from the environment (Clark and Diamond, 1997; Shiratori and Ishida, 2015).

Most *Entamoeba* gene sequences in public databases originate from species living in endothermic hosts, while relatively few derive from species living in ectotherms. To date, the latter hosts include amphibians, reptiles and insects (Silberman *et al.*, 1999; Garcia *et al.*, 2014; Clark and Stensvold, 2015; Jacob *et al.*, 2015; Kawano *et al.*, 2017). Herein, we report a new species of *Entamoeba*, isolated from the gastrointestinal tract of the fish *Monopterus albus* (the Asian swamp eel) in Chiang Rai, Thailand. We examine its morphological features using light microscopy of living and stained specimens and provide the first SSU rRNA gene sequence of an *Entamoeba* isolated from fish.

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Table 1. Primers used to amplify and sequence *Entamoeba chiangraiensis*

Primer name	Primer sequence (5′–3′)	References
RD5	ATCTGGTTGATCCTGCCAGT	Clark *et al.* (2006)
RD3	ATCCTTCCGCAGGTTCACCTAC	
ENTAGEN_F	ACTTCAGGGGGAGTATGGTCAC	Stensvold *et al.* (2011)
ENTAGEN_R	CAAGATGTCTAAGGGCATCACAG	
ENTAM1	GTTGATCCTGCCAGTATTATATG	Verweij *et al.* (2001)

Methods

Sample collection and establishment of culture

Two Asian swamp eels were purchased at a local market at Sanpong village, Phan district, Chiang Rai Province, northern Thailand. The eels were obtained at two separate times, in May and July 2018. Colonic contents were placed in modified (no mucin was added) LYSGM medium (Diamond, 1982, http://entamoeba.lshtm.ac.uk/xenic.htm) and incubated at room temperature (25–27 °C). After 24 h, sediment was transferred to fresh medium and cells were subcultured every 2 weeks. The culture has been maintained since July 2018.

Light microscopy and staining

A wet mount of live amoebae was prepared and cells were observed using Nikon inverted light microscope. Trophozoites ($n = 10$) and cysts ($n = 125$ live; $n = 125$ stained with iodine) were measured using the same microscope. For a more detailed view of the cells, iron hematoxylin staining was performed by the Diagnostic Parasitology Laboratory, London School of Hygiene and Tropical Medicine. Stained cells were observed with a Leica DMRB microscope fitted with a DFC 420 camera.

DNA extraction, amplification, purification and sequencing

Total genomic DNA was extracted from the culture using an AccuPrep® Genomic DNA Extraction Kit (Bioneer, South Korea, catalog No: K-3032) according to the manufacturer's specifications. Polymerase chain reaction (PCR) using the broad specificity primers RD5 and RD3 was used to amplify almost the entire SSU rRNA gene (Table 1). Emerald Amp® GT PCR Master Mix for PCR reactions was obtained from TaKaRa Bio USA, Inc. Cycling conditions were as follows: initial denaturation at 94 °C for 3 min, followed by 40 cycles of: denaturation at 94 °C for 1.3 min, annealing at 60 °C for 1 min and extension at 72 °C for 2 min, ending with a final extension of 10 min at 72 °C.

The resulting PCR products were purified from gels with the GeneJET Gel Extraction Kit (Thermo Scientific; Wardmedic, Thailand) according to manufacturer's specifications. Samples were sequenced with RD5 and RD3 primers, along with ENTAM1, ENTAGENF and ENTAGENR (Table 1).

Phylogenetic analysis

The chromatogram quality of raw reads was checked individually with Sequencher software and ambiguous bases from the ends were removed. Sequences were combined into contigs and checked against the NCBI nr database, where they were identified as *Entamoeba*. A dataset was assembled including the newly derived sequences along with sequences spanning the breadth of molecular diversity of *Entamoeba*. In total, 90 sequences were used. Sequence alignment was performed on the EBI online platform (https://www.ebi.ac.uk/Tools/msa/mafft/) using MAFFT v.7.394 (Katoh and Toh, 2010). Ambiguously aligned positions were removed using Trimal v.1.3 (Capella-Gutierrez *et al.*, 2009) available on the online platform Phylemon 2.0 (http://phylemon.bioinfo.cipf.es). After trimming 1434 sites remained. Maximum likelihood analysis was conducted using RAxML v.8 (Stamatakis, 2006) on the online platform CIPRES Science Gateway (http://www.phylo.org/index.php/). For ML analysis, the general time reversible + Γ model of nucleotide substitution was employed as dictated by jModelTest v.2.1.10 using the Akaike criterion. Bootstrap support was computed from 1000 bootstrap replicates.

Results

Culture, light microscopy and phylogenetic analysis

Colonic gut contents were inoculated into modified LYSGM, a medium widely used for xenic cultivation of *Entamoeba* species, and incubated at room temperature overnight. No live amoebae or cysts were observed in any tubes incubated at 37 °C, indicating that this species does not survive at that temperature.

The trophozoite of the amoeba is longer than it is wide (Fig. 1, Fig. 2C and D). Length is 40–50 μm (mean 44.31 μm) when the amoeba swims, but when it glides on the slide it ranges from 30 to 40 μm, while width ranges from 9 to 13 μm (mean 11.18 μm). The cell changes shape slowly while in motion and has a single prominent pseudopodium, while the posterior end is smooth with no obvious uroid (Fig. 1, Fig. 2C and D). The granuloplasm has multiple vesicles while the hyaloplasm is narrow (Fig. 1A). Unstained spherical cysts range from 10.0 to 17.50 μm in diameter (mean 14.15 μm; ±1.42 standard deviation; ±0.13 standard error). Stained cysts range from 10.0 to 17.50 μm in diameter (mean 13.75 μm; ±1.54 standard deviation; ±0.14 standard error). All observed cysts in both live and stained samples were uninucleate (Fig. 2A and B), with the exception of a single stained example where it looked like there were two nuclei. Large, prominent glycogen vacuoles were present in both live and stained cysts, indicating that all observed cysts were immature (Fig. 2A and B). Therefore, we cannot state the number of nuclei per cyst definitively, as we were not able to observe mature cysts. Cysts have no distinctive appearance (Fig. 2A and B).

The size of the nucleus in both cysts and trophozoites ranges in diameter from 2.5 to 7.5 μm (mean 3.97 μm; ±1.46 standard deviation; ±0.13 standard error) and is generally found in the anterior half of the trophozoite. The trophozoite nucleus has a karyosome that has the appearance of a cluster of granules (Fig. 2C and D). Karyosome size is variable depending on how tightly the granules cluster. Chromatin forms a delicate, even lining along the inner membrane of the nucleus (Fig. 2D). Unlike many other *Entamoeba* species, there are no clearly visible clumps of peripheral chromatin. Radial spokes are present in the nucleus joining the karyosome to peripheral chromatin (Fig. 2C).

The SSU rRNA gene sequences of the two isolates are nearly complete (1849 and 1856 bp). Both sequences have been deposited in GenBank under accession numbers MK652887 and MK652888. The overall topology of the phylogenetic tree is similar to previous studies (Jacob *et al.*, 2015). The tree is artificially rooted to the clade containing the cockroach sequences. These were the earliest diverging *Entamoeba* sequences in the eukaryotic supergroup tree of Kawano *et al.*, 2017. The new SSU rRNA gene sequences are sister to those from *E. invadens* and this relationship has maximum bootstrap support (Fig. 3). The genetic distance between the new sequences and *E. invadens* sequences ranges from 3.4 to 3.8% (Table S1). All observed nucleotide differences (including insertion and deletion events) are taxon-specific.

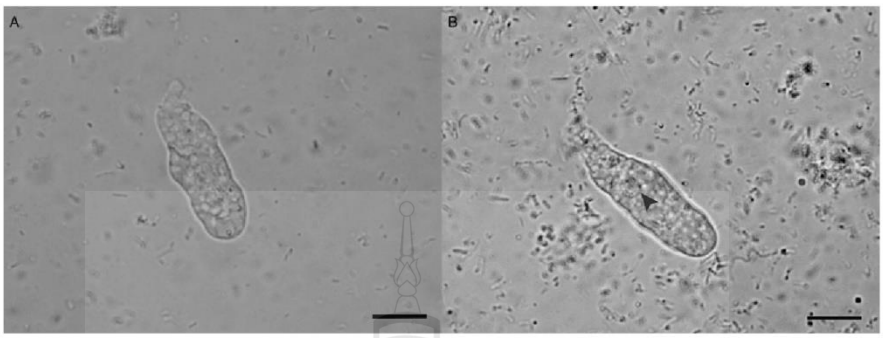

Fig. 1. Light micrographs of living trophozoites of *Entamoeba chiangraiensis* n. sp. Arrowhead indicates the nucleus. Scale bar = 25 μm.

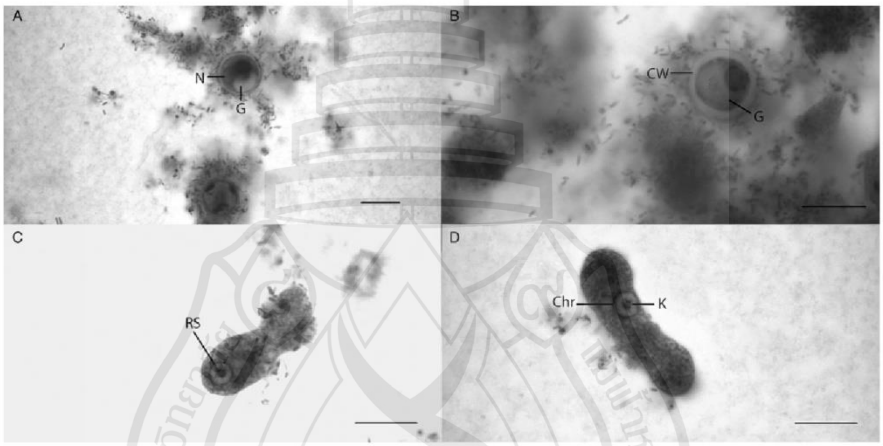

Fig. 2. Light micrographs of trophozoites and cysts stained with iron hematoxylin. (A and B) Stained cysts. N, nucleus; G, glycogen vacuole; CW, cyst wall. (C and D) Stained trophozoites. RS, radial spokes connecting karyosome to peripheral chromatin; Chr, peripheral chromatin forming an even fine lining around nuclear membrane; K, karyosome consisting of granules. Scale bar = 10 μm.

Intraspecific genetic divergence for the new amoeba and *E. invadens* is 0 and 0.4%, respectively. These sister species are in a clade that also includes *E. ranarum* and an unnamed *Entamoeba* sp., both from amphibian hosts. All members of this clade have been isolated from ectothermic hosts. This clade also has maximum bootstrap support.

Taxonomic Summary

Amoebozoa Lühe 1913, emend. Cavalier-Smith 1998
Archamoebae Cavalier-Smith 1983
Entamoebidae Chatton 1925, emend. Cavalier-Smith 1993
Entamoeba Casagrandi & Barbagallo 1895
Entamoeba chiangraiensis n. sp. Jinatham, Clark & Gentekaki 2019

Diagnosis: Amoeba inhabiting the gut of *Monopterus albus* (Asian swamp eel). Trophozoite is much longer than it is wide; length in motion is 30–50 μm, width 9–13 μm. A trailing end is smooth and devoid of visible uroid processes. Cysts are spherical, appearing smooth and thick-walled. Immature cysts have a single nucleus and a prominent glycogen vacuole, which often obscures the nucleus. Cyst diameter is 10.0–17.5 μm (mean 14.15 μm; ±1.42 standard deviation; ±0.13 standard error), nucleus 2.5–7.5 μm (mean 3.97 μm; ±1.46 standard deviation; ±0.13 standard error). There is a karyosome composed of granules. Chromatin is evenly distributed around the inner nuclear membrane, forming a thin, uniform lining. Radial spokes connect the karyosome to the peripheral chromatin.

Etymology: the epithet *chiangraiensis* refers to Chiang Rai province, Thailand, in which the organism was isolated

Host: *Monopterus albus*

Type location: isolated from the gut of Asian swamp eel, Sanpong, Phan, Chiang Rai, Thailand

Type material: permanent slide stained with iron-hematoxylin was deposited in the Smithsonian Museum under accession number USNM 1484171.

Type sequence: GenBank accession number MK652887

Fig. 3. Maximum likelihood phylogenetic tree inferred from 90 SSUrRNA sequences and 1434 sites. The tree is artificially rooted to cockroach-derived *Entamoeba* sequences. Newly generated sequences are depicted in bold lettering. Numerical values indicate bootstrap support. Only values above 70 are shown. Full circles represent maximum bootstrap support. Clades in red consist of sequences exclusively from ectothermic hosts.

Discussion

Like all members of the genus *Entamoeba*, the new species has a nucleus with the characteristic 'ring and dot' appearance corresponding to peripheral chromatin and central karyosome (Clark and Stensvold, 2015). *Entamoeba chiangraiensis* n.sp. was isolated twice from the Asian swamp eel, *Monopterus albus*, which inhabits rivers across Southeast Asia. Only a few species of *Entamoeba* from fish have been documented: four from marine hosts and three from freshwater (Table 2 and references therein). Molecular data for any of these species is absent.

Pathogenicity of the new species is unknown. Only a few species of *Entamoeba* are definitively pathogenic based on histology evidence. These are *E. histolytica*, a human pathogen, *E. nuttalli*, a pathogen of non-human primates, *E. invadens*, a reptile pathogen and *Entamoeba* sp., a toad pathogen (Clark and Stensvold, 2015; Shilton *et al.*, 2018). Microscopic examination of *E. chiangraiensis* cells immediately after sample collection did not reveal ingestion of red blood cells, suggesting that the species is commensal rather than invasive. Nonetheless, to definitively determine pathogenicity further studies will be needed, including histology of infected fish to detect whether *E. chiangraiensis* invades host tissue.

We observed a single nucleus in cysts of the new species. However, the number of nuclei in mature cysts remains undetermined as cysts degenerated before reaching maturity. In the literature, the number of nuclei in cysts of Entamoebae from fish varies from one to four (Table 2 and references within). Species of *Entamoeba* from other ectothermic hosts commonly have four nucleated cysts, although octo-nucleated cysts have been observed in some reptiles, including *E. barreti* from a snapping turtle (Taliaferro and Holmes, 1924).

The host range of our and other species of *Entamoeba* from fish is unknown. We screened a number of fish inhabiting the same environment as the Asian swamp eel (Synbranchiformes) including: *Anabas* sp. (Anabatiformes, $n = 3$), *Tilapia* sp. (Cichliformes, n-5), *Trichogaster* sp. (Anabatiformes, $n = 3$), *Trachinocephalus* (Aulopiformes, $n = 2$) and Siluriformes (Siluriformes, $n = 4$). Our examination included both microscopy and a molecular survey using combinations of the primers described in the methods

Table 2. Species of *Entamoeba* isolated from fish

Species	Host-salinity, location	# of cyst nuclei	Cyst diameter (μm)	References
Entamoeba chiangraiensis	Asian swamp eel (*Monopterus albus*), freshwater, Thailand	Uncertain	10.0–17.5	This report
Entamoeba ctenopharyngodoni	Carp, freshwater, China	1–4	7.8–10.4	Chen (1955)
Entamoeba gadi	Pollock (*Pollachius virens*), marine, USA	1–2	6.0–11.8	Bullock (1966)
Entamoeba molae	Ocean sunfish (*Mola mola*), marine, USA	1	Not observed	Noble and Noble (1966)
Entamoeba nezumia	Macrourid fish (*Nezumia bairdi*), marine, Greenland	1–4	7.7	Orias and Noble (1971)
Entamoeba pimelodi	Catfish (*Pimelodus clarias*), freshwater, Brazil	1	Not mentioned	da Cunha and Penido (1926)
Entamoeba salpae	Fish (*Box salpa* syn. *Sarpa salpa*), marine, France	4	Not mentioned	Alexeieff (1912)
Entamoeba synodontis[a]	Catfish (*Synodontis schall*), freshwater, Egypt	Uncertain	Uncertain	Imam *et al.* (1987)

[a]Description is incomplete in the original text

section. Intestinal contents from all fish were placed in the same culture medium in an attempt to grow amoebae. We were unable to find *Entamoeba* in any of the other hosts using any of the methods described. Although we tried to be as inclusive as possible in our screening, we cannot exclude the possibility that *E. changraiensis* might also inhabit the gut of fish that we have not examined. Host ranges of many *Entamoeba* species remain incompletely known, but they keep expanding. For instance, *E. coli* has traditionally been reported from humans and non-human primates but is now known in rodents (Clark and Stensvold, 2015). Nonetheless, it seems likely that body temperature will pose a constraint on host range, as Entamoebae from ectotherms have not been found in endotherms and vice versa. *Entamoeba moshkovskii* is a notable exception, having been found in both reptiles and mammals (Garcia *et al.*, 2014); it seems to be the only species of *Entamoeba* that has crossed the ectotherm/endotherm barrier. Within ectotherms, *Entamoeba* species show host specificity at the higher level of classification. Thus, reptilian isolates have never been isolated from amphibians and vice versa.

Entamoeba SSU rRNA gene sequences that have been detected exclusively in ectothermic hosts are diverse and dispersed across the phylogenetic tree, forming four distinct clades. The first clade comprises *E. chiangraiensis*, *E. invadens*, *E. ranarum* and an unnamed *Entamoeba* sp. (MH890608) from a toad. The latter represents only the second amphibian-derived *Entamoeba* sequence. The SSU rRNA gene sequences from two eels sampled at two separate time points were identical, indicating low intraspecific diversity of this gene in *E. chiangraiensis*. This is similar to *E. invadens*, whose SSU rRNA gene sequences also display a high degree of genetic similarity, even when isolated from different hosts and from different countries (Jacob *et al.*, 2015). The new species groups together with *E. invadens*. When comparing their SSU rRNA sequences, the genetic distance is a little below 4%, almost 4-fold than that between *E. histolytica* and *E. dispar*. The second clade contains several variants of *E. terrapinae* derived from aquatic turtles (Garcia *et al.*, 2014). The third clade contains *Entamoeba insolita*, along with *Entamoeba* RL5 from tortoise and *Entamoeba* RL6 from iguana. These organisms are each represented by a single sequence (Silberman *et al.*, 1999; Stensvold *et al.*, 2011). Finally, the fourth clade consists of numerous sequences of *Entamoeba* from cockroaches (Kawano *et al.*, 2017). In their study, Kawano *et al.* (2017) examined 186 cockroaches and found Entamoebae in 134. In their phylogenetic analyses, cockroach-derived sequences formed a distinct clade with nine separate groups within. This strongly hints at the presence of a vast diversity of *Entamoeba* that has yet to be uncovered. It seems likely that screening of additional hosts, especially ectotherms, will reveal an ever greater number of novel *Entamoeba* species.

Supplementary material. The supplementary material for this article can be found at https://doi.org/10.1017/S0031182019000775.

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Conflict of interest. None.

Ethical standards. No animals were sacrificed specifically for this work. Asian swamp eel is a popular food in Thailand and can be purchased at local markets. Intestinal contents were obtained from eels that had been purchased for food consumption. Permission and approval for obtaining such contents were obtained from the Mae Fah Luang University Animal Care and Use Committee (protocol no. AR01/62).

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Prototheca bovis, a unicellular achlorophyllous trebouxiophyte green alga in the healthy human intestine

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Abstract

Introduction. *Prototheca* species are non-photosynthetic trebouxiophyte algae ubiquitously distributed in nature and can be found in sewage and soil. This microbial eukaryote causes human protothecosis in immunocompromised individuals. Thus, *Prototheca* presence in the stool of individuals without gastrointestinal symptoms has been reported only rarely.

Hypothesis/Gap statement. There is an absence of detailed characterization of human *Prototheca* isolates.

Aim. The aim of this study was to perform morphological and molecular characterization of *Prototheca* isolates obtained from human stool.

Methodology. *Prototheca* was isolated from faecal samples of four individuals living in a rural area in Thailand. A combination of bioimaging along with molecular and bioinformatics tools was used to characterize the four strains. The growth rate was tested using four media and three temperature conditions. Phylogenetic analysis using the small subunit ribosomal RNA (SSU rRNA) and cytochrome b (*cytb*) was also performed.

Results. Static and live microscopy demonstrated the various life stages of *Prototheca* and its major defining cellular characteristics. An optimized DNA extraction methodology that improves DNA yield is provided. Partial fragments of the SSU rRNA and *cytb* genes were obtained. Phylogenetic analysis placed all four strains in the clade with *Prototheca bovis*. More broadly, *Prototheca* was not monophyletic but split into at least two distinct clades instead.

Conclusion. The results represent the first molecular characterization of *Prototheca* in Thailand. The study provides insight into transmission dynamics of the organism and potential caveats in estimating the global prevalence of *Prototheca*. These will spearhead further investigations on *Prototheca* occurrence in rural areas of both industrialized and developing nations.

INTRODUCTION

Studies on human enteric organisms in Thailand have focused primarily on viral and bacterial pathogens and parasitic worms, such as *Opisthorchis*, *Taenia*, and *Enterobius* [1–3]. With the exception of *Blastocystis*, *Cryptosporidium*, *Entamoeba*, and *Giardia*, other microbial eukaryotes are usually overlooked [4–7]. One such example is *Prototheca*, a unicellular achlorophyllous trebouxiophyte green alga [8]. *Prototheca* has been isolated from a broad range of animal species, including cats, dairy cattle, rats, and swine [9, 10]. In dairy cattle, the organism causes mastitis [11–13]. Skin and soft tissue infections are seen in dogs and cats [14, 15]. Five species of *Prototheca* are known to infect humans and/or other animals: *P. blaschkeae*, *P. cutis*, *P. wickerhamii*, *P. ciferrii* (previously known as *P. zopfii* genotype 1) and *P. bovis* (previously known as *P. zopfii* genotype 2) [16–18]. The latter is of special interest as it has been found in both

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Abbreviations: *cytb*, cytochrome b; LV, low viscocity; MEA, malt extract agar; NA, nutrient agar; PDA, potato dextrose agar; PDB, potato dextrose broth; SSU rRNA, small subunit ribosomal RNA; TEM, transmission electron microscopy.
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One supplementary file and four supplementary figures are available with the online version of this article.

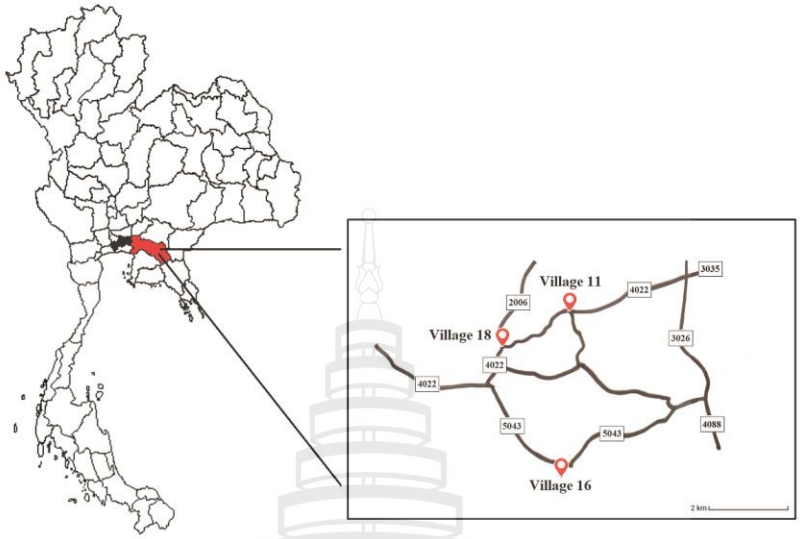

Fig. 1. Sampling locations in villages 11, 16 and 18, Chachoengsao Province, Thailand.

animals and humans. The disease caused by *Prototheca* is known as protothecosis [18–20].

Prototheca bovis and *P. wickerhamii* are currently the two most common etiological agents of the disease [19, 21, 22]. *Prototheca* infections have been reported in Europe, Asia, North America, and Africa [23]. Nonetheless, the number of human cases is sparse: less than 200 cases of human protothecosis have been reported worldwide [20, 24]. Human protothecosis has three main manifestations: cutaneous lesions, olecranon bursitis, and disseminated or systemic infection accompanied by varying symptoms [25]. Cutaneous lesions are the most common manifestation of human protothecosis. *Prototheca* has only very rarely been isolated from the human intestine [26].

While performing a survey for eukaryotic microbes in a rural area of Thailand, we identified four potential cases of human intestinal protothecosis. Using a combination of tools from culturomics, cellular and molecular biology, and phylogenetics we characterized the isolates as *Prototheca bovis*.

METHODS

Ethics statement

The Human Ethics Committee of Phramongkutklao College of Medicine approved the collection of faecal samples from Thai volunteers (License approval number S053q/58).

Human subjects and sample collection

Human volunteers were recruited as part of a large-scale parasitological survey in Chachoengsao Province, Thailand. All volunteers were Thai nationals and lived in the province at the time of collection (Fig. 1). Faecal samples were randomly collected from 98 volunteers living in three villages (villages 11, 16, and 18), who did not have diarrhoea at the time of collection. Sterile collection kits containing a plastic container, gauze, and spatula were distributed to all volunteers. Small amounts of each faecal sample were introduced in two separate tubes containing HL-5 and LYSGM [27] media (http://entamoeba.lshtm.ac.uk/xenic.htm). Upon transfer to the laboratory, all samples (98 tubes of HL-5 and 98 tubes of LYSGM) were incubated at 37 °C.

Culture conditions

The cultures in HL-5 media and LYSGM were monitored daily using light microscopy starting from day five post-inoculation. *Prototheca* cells were observed in samples from four volunteers in both media. One millilitre of *Prototheca* positive samples was sub-cultured in fresh media. After 24 h, 10 µl of each *Prototheca* culture were streaked on Petri plates containing potato dextrose agar (PDA) and sealed with parafilm. Colonies appeared within 24 h. A single colony was then picked from each of the four cultures and transferred into fresh PDA media. Cultures were also established in potato dextrose broth (PDB). Pure cultures of all four isolates were

also established in blood agar, nutrient agar (NA), and malt extract agar (MEA) to examine macroscopic colony characteristics. The PDA plates were incubated at 25 °C, 37 °C, and 40 °C for 3 days to observe the growth pattern of *Prototheca* isolates. Photographs were taken on day three.

Prototheca cells were counted using a haemocytometer and seeded at a density of 5000 cells per well of a 12 well plate (Greiner) containing 1 ml of PDB to generate video stills. The plate was mounted on a JuLiStage Real-Time Cell History Recorder, inside an incubator at 37 °C. The JuLiStage system was set to image the wells every minute until the cells in the field of view reached confluency using a 10× zoom lens. Image captures were then assembled into a video using the JuLiStage proprietary software. Image stills were taken using the snapshot function on the VLC media player (VideoLAN). Image editing was done using the Graphical Image Manipulation Program.

Microscopy

Faecal smears were prepared by diluting faecal matter with sterilized bottled drinking water. Micromorphological characteristics of *Prototheca* cells were observed by diluting PDB culture with bottled water. A Nikon 80i compound microscope equipped with a Nikon DS-Ri2 camera was used. Transmission electron microscopy (TEM) was carried out to characterize key morphological features in detail. Briefly, cells of *Prototheca* were centrifuged at 300 ***g*** and resuspended in 2.5% glutaraldehyde, 100 mM sodium cacodylate buffer (pH 7.2), and fixed overnight at 4 °C. Samples were washed with cacodylate buffer twice for 10 min and then post-fixed in 1% osmium tetroxide in cacodylate buffer for 1 h at room temperature. Samples were then washed twice in distilled water for 10 min and then dehydrated through a graded ethanol series of 50, 70, 90% at 10 min per step and then three times for 10 min in 100% ethanol. This was followed by two 10 min incubations in propylene oxide, followed by 30 min in 50:50 propylene oxide:agar low viscosity (LV) resin. Samples were incubated twice for 2 h in freshly prepared agar LV resin and then spun down in BEEM capsules. The resin infiltrated samples were polymerised at 60 °C for 24 h. Sections of 70 nm were cut on a Leica EM UC7 ultramicrotome and collected on 400 mesh copper grids. Sections were counterstained in 4.5% uranyl acetate in 1% acetic acid for 45 min and Reynolds lead citrate for 7 min. Sections were imaged in a JEOL 1230 Transmission Electron Microscope operated at 80 kV, and images were captured with a Gatan One view digital camera.

DNA extraction, PCR and Sequencing

Genomic DNA was extracted from pure cultures using a Qiagen DNA stool mini kit (Qiagen, Hilden, Germany) with the following modification. Briefly, during the lysis step, 250 mg of 0.5 mm zirconia beads and 20 µl of 10% SDS were added along with the lysis buffer provided by the kit. Polymerase chain reaction (PCR) was performed using EmeraldAmp GT PCR Master Mix (TaKaRa Bio USA, Inc.). Genomic DNA was also extracted from the faecal samples of the four *Prototheca* positive individuals. A fragment of 1550 bp of the small subunit ribosomal RNA (SSU rRNA) gene was amplified using DNA extracted from pure cultures with the NS1F: 5′-GTAGTCATATGCTTGTCTC-3′ [28] and proto18S-4r: 5′-AGCACACCCAATCGGTAGGA-3′ primers [17]. The PCR conditions were as follows: denaturation at 95 °C for 3 min, 35 cycles with denaturation at 95 °C for 2 min, annealing at 55 °C for 90 s, extension at 72 °C for 2 min, and a final extension at 72 °C for 10 min. DNA extracted from the stool of *Prototheca* positive individuals was used in an attempt to amplify a fragment of 430 bp of the SSU rRNA using the proto18S-4f: 5′-GACATGGCGAGGATTGACAGA-3′ and proto18S-4r primers. The PCR conditions were as follows: denaturation at 95 °C for 3 min, 35 cycles with denaturation at 95 °C for 90 s, annealing at 55 °C for 1 min, extension at 72 °C for 2 min and a final extension at 72 °C for 10 min. All amplified products were purified with GeneJET Gel Extraction Kit (Thermo Scientific) and sent for bidirectional sequencing at U2Bio (Korea). A fragment of 600 bp of the *cytb* gene was amplified from DNA obtained from pure cultures using the cytb-F1:5′-GyGTwGAACAyATTATGAGAG-3′ and cytb-R2:5′-wACCCATAArAArTACCATTCwGG-3′ primers [29]. Sequences have been deposited in GenBank under the accession numbers MT920919, MW228085-87 (SSU rRNA), and MZ209407-MZ209410 (*cytb*).

Phylogenetic analysis

The forward and reverse sequencing chromatograms were combined into contigs using CLC Main Workbench v.8 (Qiagen). The assembled sequences of SSU rRNA and *cytb* genes were used as queries to perform BLAST searches against GenBank to exclude contamination and collect additional sequences of *Prototheca* and other green algae. Two datasets were assembled, one for SSU rRNA and one for *cytb*. The SSU rRNA dataset contained 203 sequences spanning the diversity of trebouxiophyte algae, and including reference and type sequences of *Prototheca* was assembled and aligned using MAFFT v.7.394 [30]. The *cytb* dataset contained 108 sequences. Ambiguous and poorly aligned regions were removed using Trimal v.1.3 [31] available on the online platform Phylemon 2.0 (http://phylemon.bioinfo.cipf.es). Following trimming, 1117 and 597 sites remained in the SSU rRNA and *cytb* datasets, respectively. Maximum likelihood trees were constructed using RAxML v.8 [32] available on CIPRES Science Gateway v. 3.3 (http://www.phylo.org/index.php/). The general time-reversible + Γ model of nucleotide substitution was used. Bootstrap support was assessed from 1000 bootstrap replicates.

RESULTS

Isolation and culturing of *Prototheca bovis* from four human stool samples

Culture media containing faecal samples of 98 volunteers were examined using a compound microscope. *Prototheca* was found in the stool of four volunteers. Faecal smears of the *Prototheca* positive stool samples provided additional confirmation of its presence (Fig. S1, available in the online version

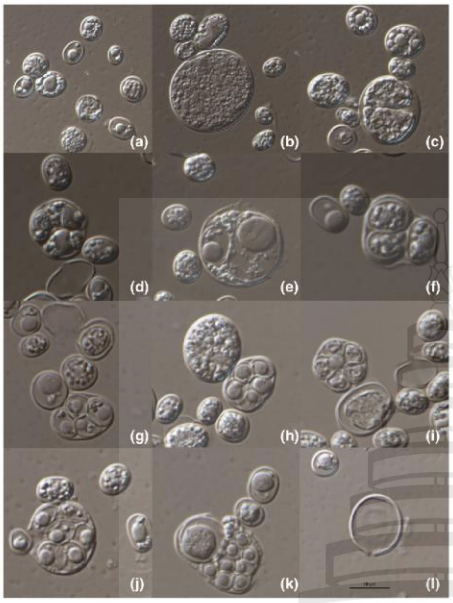

Fig. 2. Details of *Prototheca sp.* cells incubated on PDA medium at 37 °C for 72 h. *Prototheca sp.* sporangiospores (a); sporangium just before first cell division, granular content clearly visible (b); sporangia at various stages of division and released sporangiospores (c-i); sporangiospore exiting bursting sporangium (j); mature sporangium containing eight sporangiospores (k); Remnant cell wall of a bursting sporangium (l). Scale bar: 10 µm.

of this article). Colonies grew on all four media (PDA, MEA, NA, and blood agar). Cells appeared within 12 h and dense colonies appeared on the nutrient-rich PDA, MEA, and blood agar plates, while colonies were smaller and not as dense in the nutrient-poor NA (Fig. S2). Colonies were yeast-like and with smooth edges. In PDA, MEA and blood agar, colonies were pale white to cream-white, 2 to 3 mm in diameter, while those in NA were one-third of the size at the same time of incubation (day 3). *Prototheca* colonies were also incubated at different temperatures to observe growth. At 25 °C, colonies of *Prototheca* on PDA were smaller than those at 37 and 40 °C on day 3 (Fig. S3).

Prototheca bovis growth pattern

The algal growth, maturation, and division were recorded live. Cells seeded in PDB media were monitored until the field of view was confluent. Upon maturation, cells (sporangia) ruptured to yield eight daughter cells (sporangiospores) (File S1). Under the growth conditions used herein, newly divided daughter cells reached maturity and divided anew in five and a half hours (File S1). A crowded environment appeared to affect cell size, as when the field of view reached confluency, most of the newly divided cells were smaller in size compared to cells of previous generations.

Microscopic investigations highlights basic cell features

All four isolates formed sporangia seen at various stages of multiplication containing two, four, or eight sporangiospores. Sporangia with more than eight sporangiospores were not observed at any point. Sporangiospores arranged in morulae (flower petal formation) were commonly observed (Fig. 2). Sporangiospores were 7.0–16.0 µm long×6.0–12.5 µm wide. Thick septa were present between sporangiospores within the sporangia. The thick cell wall characteristic of *Prototheca* was clearly visible.

Transmission electron microscopy was used to obtain high-resolution images of *Prototheca* cells. Various life stages of *Prototheca* and its subcellular organelles were observed (Fig. 3). These included a cell membrane surrounded by a thick cell wall, canonical mitochondria (numerous cristae), remnant plastids with double membranes, filled with starch granules, and dense lipid droplets of various sizes dispersed in the cell (Fig. 3).

Sequence and phylogenetic analyses

Prototheca sequences were successfully obtained only for the fragments amplified from the DNA obtained from pure cultures. The sequences from DNA extracted from stool samples were from edible plants. The newly generated SSU rRNA sequences were identical to each other. Two separate phylogenetic trees were inferred from SSU rRNA (Fig. S4) and *cytb* (Fig. 4) gene sequences. In the SSU rRNA tree, *Prototheca* was not monophyletic, as it split into three clades with *Helicosporidium* grouping within. These clades were: (1) a clade containing most *Prototheca* species; (2) a clade containing *P. miyajii* and *P. cutis*, which grouped as sister to *Helicosporidium*, albeit with low support; and (3) the *P. xanthoriae* clade, which grouped as sister to *Auxenochlorella*. The latter clade contained two *P. xanthoriae* sequences, which grouped separately from the typed sequence of this species, which groups within clade 1. The four newly identified strains nested within *P. bovis* sequences along with a single sequence of *P. xanthoriae* in clade 1. In the *cytb* gene tree, *Prototheca* was also not monophyletic, with strains of *Chlorella*, *Helicosporidium*, and *Auxenochlorella* grouping within. The *Prototheca* sequences were distributed into the same nine previously defined clusters (I-IX), with each cluster representing a separate species [29]. The newly generated sequences grouped with *P. bovis*; thus, we have designated them as such.

DISCUSSION

Prototheca is likely transmitted to humans by an animal or environmental reservoir even though these sources have yet to be identified [16]. Human protothecosis manifests

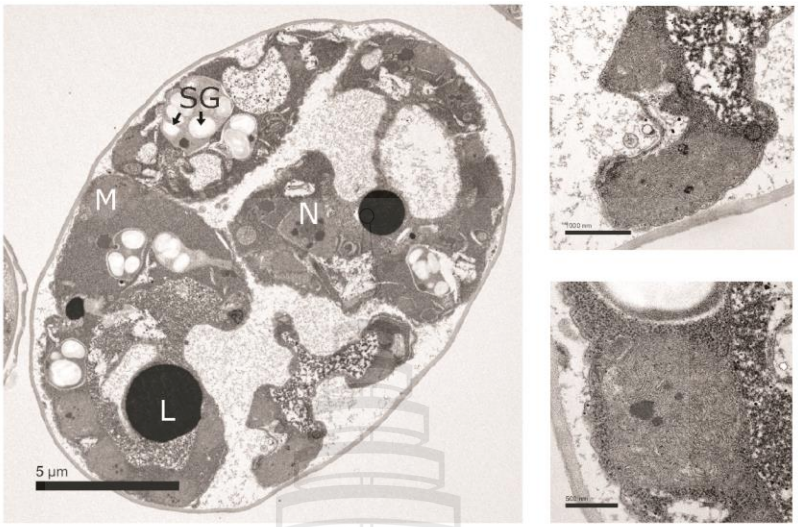

Fig. 3. Transmission electron microscopy depicting structural features commonly associated with *Prototheca* species: lipid droplets (l), nucleus (n), mitochondria (m), starch granules (SG) and a thick cell wall (Panel A). Close up of mitochondria located near the cell walls (Panels B and C).

as localized infection of the skin, olecranon bursitism and disseminated infection [20, 24, 33]. It has been proposed that immunocompromised individuals and those with underlying conditions are more susceptible to the disease [34, 35]. The two most common species of *Prototheca* causing the human infection are *P. bovis* and *P. wickerhamii*. The latter is the principal etiological agent of human protothecosis, whereas *P. bovis* commonly causes disease in other animals [10, 25]. Workers in rice paddies, fishermen, farmers, handlers of raw seafood, and aquarium staff are at risk of exposure to *Prototheca* [16]. Nonetheless, only a few studies from countries where these jobs are common, including South and Southeast Asian countries. In Thailand, *Prototheca* has been reported only rarely [36, 37].

Traditionally, protothecosis diagnosis has focused on microscopic observation and physiological/biochemical tests of the isolated organism or direct examination of affected tissues [24]. Nonetheless, neither of these methods can accurately identify *Prototheca* to the species level. Molecular characterization using SSU rRNA and *cytb* genes has greatly facilitated species identification [29, 38]. Herein we isolated four strains of *Prototheca* from faecal samples of Thai volunteers living in the rural area of Chacheongsao. Phylogenetic analysis of the SSU rRNA and *cytb* genes placed all four isolates in the *P. bovis* clade (Figs 4 and S4). This species is associated with bovine mastitis (in the form of persistent udder inflammation) and infections of companion animals [17, 22, 39, 40]. Nevertheless, there have been occasional reports of clinical cases of human protothecosis involving *P. bovis* [41–44]. This species was previously designated as *P. zopfii* genotype two and was recently elevated to species level [29]. Given that finding *Prototheca* in human stool is extremely rare, its occurrence in four individuals raises questions about its original source, distribution, and transmission.

Pinpointing the source of *Prototheca* is challenging, as the human-to-human transmission is unknown [19]. Herein, volunteers positive for *Prototheca* lived in four separate households distributed in two different villages 15 km apart. Participants in this study spend extended periods in rice fields and have frequent contact with ruminants and poultry, whose dung is used to fertilize their vegetable gardens. The three villages' water source comprises groundwater stored in tanks composed of layers of sand and gravel filters. Rice fields, dung, and groundwater constitute habitats from which *Prototheca* has been previously isolated [16]. Hence, the alga likely came from one or all of the aforementioned sources, though it is not possible to confidently identify the source. Given these uncertainties, a comprehensive One Health approach that includes humans, other animals, and the environment in all three villages is needed in the future.

Remarkably, little is known regarding the pathogenesis and virulence of *Prototheca*. Many studies focus on infections of immunocompromised hosts, and discovery of the

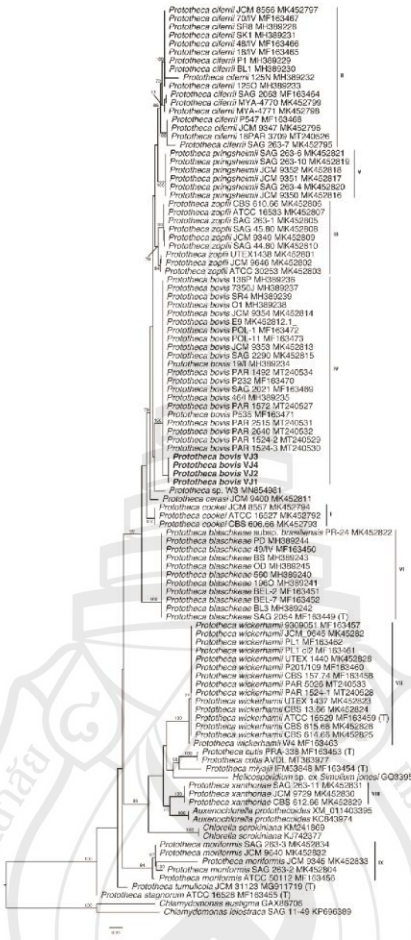

Fig. 4. Maximum likelihood phylogeny of cytochrome b inferred from 108 sequences and 597 sites. Numerical values at nodes represent maximum likelihood bootstrap support. Only values above 70 are shown. New sequences are presented in bold letters. Sequences from type specimens are represented with a (t). Roman numerals (i-ix) represent *Prototheca* species clusters as those are defined in Jaglieski *et al.* [29].

organism is often incidental [35]. Notwithstanding, its ability to survive passage through the gastrointestinal tract has been previously discussed [16]. In this study, *P. bovis* was detected in the stool of four individuals that exhibited no diarrhoea. Smears of stored faecal samples from all *Prototheca* positive volunteers revealed the presence of organisms, some of which were dividing (see Fig. S1). This finding suggests that the organism remained in the host long enough to undergo cell division. It is currently not feasible to determine whether there was an established infection as samples were collected during a large-scale survey, where only the diarrhoea status was checked. At the same time, one cannot exclude the possibility of *P. bovis* asymptomatically colonizing the hosts.

The rarity of protothecosis is a recurrent theme of literature reviews [20, 45]. Our study suggests methodological caveats as a possible cause. At the onset of the experiments, DNA yield from the newly established pure cultures of *P. bovis* was disproportionately low, given the large number of cells used for the extraction. Direct microscopic observation of cells right after lysis revealed that the vast majority of them failed to lyse. This is not surprising given the thick cell wall of *Prototheca* (see Fig. 3). After adapting various protocols, we found that using zirconia beads (0.5 mm) along with SDS (10%) improved DNA yield substantially. Microscopic examination showed that more than half of the cells had ruptured after using this lysis method. Given the difficulty of cell rupture, we started to suspect that *Prototheca* cells in the stool might not be disrupted either when using stool DNA extraction methods. To further examine this, we went back to the DNA obtained from the four original stool samples of *Prototheca* positive volunteers. To our surprise, attempts to amplify and sequence SSU rRNA and *cytb* gene fragments from these DNA samples only yielded sequences of edible plants. Collective consideration of these data strongly suggests that the alga remained intact during DNA extraction from stool samples. If this is the case, then it is highly likely that the prevalence of *Prototheca* is consistently underestimated and not considered in surveys of faecal and/or environmental eukaryotic diversity.

Herein, we performed molecular characterization of *Prototheca* from the stool of four volunteers in Thailand. Based on the potential methodological caveats discussed above, we speculate that *Prototheca* prevalence might be underestimated in human hosts. During this study, it became evident that there is a need for further studies to determine whether the organism is transient, temporary/long-term coloniser, or a pathogen. Thus, there is an urgent need to elucidate aspects of the biology and life cycle of *Prototheca*. In that vein, we outline a toolkit of techniques that can be used to study cell biology and characterize specific components of *Prototheca* in the future. Such approaches comprise a significant step towards developing this organism as a model to understand various aspects of its pathogenicity and opportunistic nature.

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Author contributions
V.J.: formal analysis, methodology, investigation, writing-original draft, writing-review and editing. D.M.C.: methodology, investigation. I.R.B.: methodology, investigation. T.V.: investigation. P.S.: investigation. S.P.: investigation. A.D.T.: methodology, investigation, resources, supervision, writing-original draft, writing-review and editing. E.G.: conceptualization, funding acquisition, methodology, investigation, project administration, supervision, writing-original draft, writing-review and editing.

Conflicts of interest
The authors declare that there are no conflicts of interest.

Ethical statement
The Human Ethics Committee of Phramongkutklao College of Medicine approved the collection of faecal samples from Thai volunteers (License approval number S053q/58) used in this study.

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Blastocystis One Health Approach in a Rural Community of Northern Thailand: Prevalence, Subtypes and Novel Transmission Routes

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Blastocystis is the most commonly found eukaryote in the gut of humans and other animals. This protist is extremely heterogeneous genetically and is classified into 28 subtypes (STs) based on the small subunit ribosomal RNA (*SSU* rRNA) gene. Numerous studies exist on prevalence of the organism, which usually focus on either humans or animals or the environment, while only a handful investigates all three sources simultaneously. Consequently, understanding of *Blastocystis* transmission dynamics remains inadequate. Our aim was to explore *Blastocystis* under the One Health perspective using a rural community in northern Thailand as our study area. We surveyed human, other animal and environmental samples using both morphological and molecular approaches. Prevalence rates of *Blastocystis* were 73% in human hosts ($n = 45$), 100% in non-human hosts ($n = 44$) and 91% in environmental samples ($n = 35$). Overall, ten subtypes were identified (ST1, ST2, ST3, ST4 ST5, ST6, ST7, ST10, ST23, and ST26), eight of which were detected in humans (ST1, ST2, ST3, ST4, ST5, ST7, ST10, and ST23), three in other animals (ST6, ST7, and ST23), while seven (ST1, ST3, ST6, ST7, ST10, ST23, and ST26) were found in the environment. In our investigation of transmission dynamics, we assessed various groupings both at the household and community level. Given the overall high prevalence rate, transmission amongst humans and between animals and humans are not as frequent as expected with only two subtypes being shared. This raises questions on the role of the environment on transmission of *Blastocystis*. Water and soil comprise the main reservoirs of the various subtypes in this community. Five subtypes are shared between humans and the environment, while three overlap between the latter and animal hosts. We propose soil as a novel route of transmission, which should be considered in future investigations. This study provides a thorough One Health perspective on *Blastocystis*. Using this type of approach advances our understanding on occurrence, diversity, ecology and transmission dynamics of this poorly understood, yet frequent gut resident.

Keywords: asymptomatic hosts, *Blastocystis*, environmental transmission, One Health, rural community, Thailand

INTRODUCTION

Blastocystis is the most ubiquitous protist inhabiting the gastrointestinal tract of human and other animal hosts (Roberts et al., 2013; Beghini et al., 2017; Stensvold and van der Giezen, 2018). Historically, diagnosis of *Blastocystis* has been based on light microscopy of fecal smears or *in vitro* cultures. The organism has four morphological forms: vacuolar, granular, amoeboid, and cyst (Tan, 2008; Parija and Jeremiah, 2013). The lack of distinct morphological features had, in the past, blurred the extent of *Blastocystis* diversity. Based on the genetic heterogeneity of the small subunit ribosomal RNA (*SSU* rRNA), *Blastocystis* is currently divided into at least 28 subtypes (STs) consisting of ST1-ST17, ST21, and ST23-ST32, all of which have been found in mammalian and avian hosts and are likely separate species (Stensvold et al., 2012; Alfellani et al., 2013b; Zhao et al., 2017; Maloney et al., 2020, 2021a,b; Stensvold and Clark, 2020; Higuera et al., 2021). Several genetically distinct *Blastocystis* lineages have also been identified in amphibian, insect and reptilian hosts, however, these are not part of the subtyping nomenclature as yet (Yoshikawa et al., 2007, 2016).

Despite earlier assumptions, subtypes do not seem to be host specific. So far, ST1-ST9 and ST12 have been reported in humans along with a single instance of ST10, ST14, and ST16 (Stensvold and Clark, 2016; Khaled et al., 2020; Osorio-Pulgarin et al., 2021). The most frequently encountered subtypes in humans are ST1-ST4, with the latter being most often reported in Europe (Deng et al., 2019; Jiménez et al., 2019; Stensvold et al., 2020). Nonetheless, the subtypes reported in humans have also been found in non-human hosts. For example, ST1 and ST3 have been identified from pigs, while ST4 is dominant in rodents (Yoshikawa et al., 2004; Stensvold et al., 2009a; Alfellani et al., 2013a; Wang et al., 2018; Betts et al., 2021).

After more than a century of research, the pathogenicity of *Blastocystis* remains questionable. Its presence in sufferers of chronic gastrointestinal illnesses including irritable bowel syndrome and inflammatory bowel disease has led to speculations about possible links to these disease states (Dogruman-Al et al., 2009a; Tan et al., 2010; Poirier et al., 2012; Cifre et al., 2018; Kesuma et al., 2019; Peña et al., 2020; Shirvani et al., 2020). However, recent studies have increasingly shown that *Blastocystis* is a frequent and stable inhabitant in the gut of hosts without gastrointestinal symptoms (Scanlan et al., 2014; Mirjalali et al., 2017; Riabi et al., 2018; Yowang et al., 2018; Kataki et al., 2019; Lhotská et al., 2020; Padukone et al., 2020). In parallel, this protist has been linked with increased bacterial richness and diversity in the human gut (Audebert et al., 2016; Chabé et al., 2017; Laforest-Lapointe and Arrieta, 2018; Tito et al., 2019; Deng et al., 2021). Therefore, a plethora of researchers now consider *Blastocystis* as a commensal rather than a pathogen.

Understanding various aspects of *Blastocystis* epidemiology will contribute significantly toward determining its pathogenicity and/or virulence of the various subtypes. To that end, elucidating routes of transmission and contributions of various sources to these routes is essential. The human-to-human, zoonotic, and waterborne transmission routes have been explored in relation to *Blastocystis* prevalence (Eroglu and Koltas, 2010; Alfellani et al., 2013b; Maloney et al., 2019). Occurrence of certain subtypes in both human and other animal hosts has led to the hypothesis that these are subtypes of zoonotic potential. For instance, ST5, typically found in pigs, and ST6, ST7 typical subtypes of avian hosts, have also been found in humans that handle them extensively (Wang et al., 2014; Greige et al., 2018). Transmission of ST8 has also been noted between non-human primates and their human zookeepers (Stensvold et al., 2009a). Waterborne transmission of *Blastocystis* has been long recognized (Li et al., 2012; Andersen and Stensvold, 2016). For instance, ST1 was identified in the water supply of a rural community in central Thailand and schoolchildren that consumed it (Leelayoova et al., 2008) and in untreated drinking water in Peninsular Malaysia (Anuar et al., 2013). Nonetheless, only scant studies simultaneously consider the contribution of more than one source to *Blastocystis* transmission.

In general, investigating transmission dynamics requires conditions that allow for uninterrupted cycling of an organism in a community. As such, developing countries comprise ideal areas to undertake these types of approaches. Herein, we undertook a One Health approach to examine *Blastocystis* epidemiology in a rural community of northern Thailand. We collected samples from humans, other animals and the environment and screened them for presence of *Blastocystis*. Data were analyzed at singular and community levels. We identified water and soil as the primary contributing sources to *Blastocystis* transmission routes in this particular community. These findings provide a multi-layered understanding of the transmission dynamics (spreading and cycling) of this controversial protist.

MATERIALS AND METHODS

Ethics Statement

The ethics committee of Mae Fah Luang University approved collection of human and animal samples used in this study (human license approval number REH60103 and animal license approval number AR01/62). Ethical rules were in accordance to the Declaration of Helsinki. Data were strictly anonymized and each sample was assigned an individual barcode.

Study Area

This study took place in a century-old rural community of 500 inhabitants in Chiang Rai Province, Thailand, between 2018 and 2019. The province is located in northern Thailand and borders Myanmar (**Figure 1**). The area of study is located across a river and villagers feed mainly on fish, vegetables and sticky rice. All residents are Thai nationals with no travel history of going abroad. There has been no immigration in the community for the last 20 years. The distance from the closest urban center is 20 km.

Sample Collection

A summary of the methodology used is provided in **Figure 2**.

Human Fecal Samples

Fecal samples were collected from 45 Thai adults. Each participant was provided with a sterile sampling kit containing

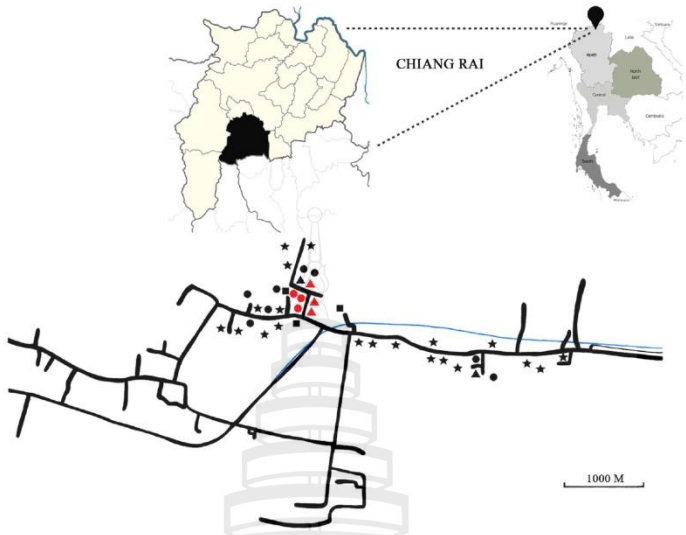

FIGURE 1 | Top right panel: Map of Thailand. Black pin has been placed on Chiang Rai Province. Top left panel: Close-up of Chiang Rai Province (in pale yellow) and the district where sampling took place (in black). Bottom panel: Detail of area of collection used in this study. Geometrical shapes represent households. Stars: Only human stool was collected. Triangles: human stool, animal stool and water were collected. Squares: Human and animal stool was collected. Circles: Human stool and water were collected. Red shapes indicate households, where stool samples were collected from all members.

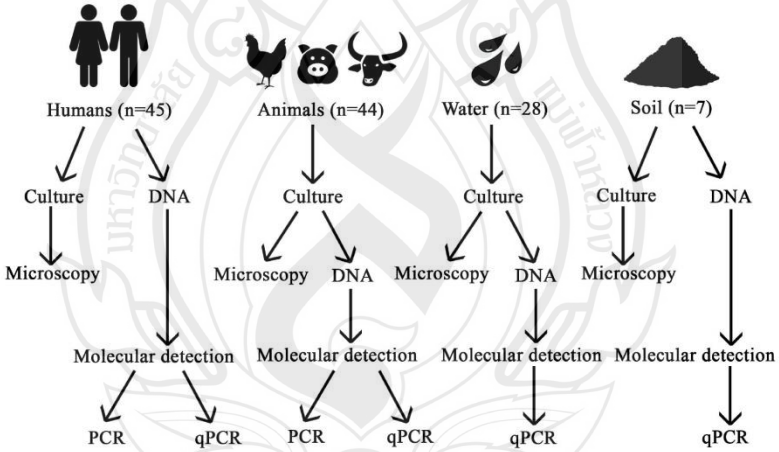

FIGURE 2 | Flow chart of the methodology used in this study.

collection container, gauze and spatula. Volunteers did not suffer from gastrointestinal diseases and had no gastrointestinal symptoms at the time of sampling. Samples were collected from 39 households, six of which housed families. A family was defined as a group of at least two people living under the same roof.

Other Animal Fecal Samples

Fecal samples were collected from 44 animals including chickens, buffalo and pigs. These animals are representative of the livestock present in the community. Several stray dogs wander freely around the community and cannot be assigned to an owner, hence these were not sampled. The animals from which samples were obtained could be traced to specific households. Chickens (n = 34) were free-range and lived in tight proximity to the household, while buffalo (n = 4) and pigs (n = 6) were housed further away from the house. Animals did not have diarrhea or blood in their stool at the time of sampling.

Water Samples

Water in the area was surveyed to investigate the possibility of an environmental reservoir of *Blastocystis*. A total of 28 water samples were collected, 17 of which were from rain collection vessels (**Supplementary Figure 1**). These are cement containers (∼ 2 m in height) present in most houses. Most of the water comes from direct rain run-off from the roof of the house. A pipe directly connects the roof to the container. Cotton plugs serve as filters to catch leaves and wood debris. A lid rests over the containers most of the time. This water is used for drinking and cooking. The containers are washed once a year during the dry season. A tap is located at the bottom of each container. One liter of water was taken from the tap of each container. Water from all containers was turbid at visual inspection.

Three samples of 1 L each were collected from the single community supply water-dispensing machine (**Supplementary Figure 2**). The water comes from a waterfall, filtered and dispensed from the machine into 10 L containers. This water is used for drinking and cooking. The containers are washed with pressurized water regularly. We sampled three of those containers.

Two samples of 1 L each were collected from the water treatment facility, from which water is distributed to every household through pipes (**Supplementary Figure 3**). The water is taken directly from the river and occasionally treated with chlorine. This water is used for bathing and watering plants (edible and non-edible).

One sample was collected from the community water tower (**Supplementary Figure 3C**). The water from the tower comes from the water treatment facility. The bottom of each tower is lined with layers of sand and gravel, which serve as additional filters. Villagers can get their water through a tap located at the bottom of the tower. The water is used mostly for cooking and bathing and rarely for drinking. A 1 L sample was taken from the tap and the water was turbid at visual inspection.

Two samples of 1 L each were collected from the river stream, which is densely grown with morning glory plants (*Ipomoea aquatica Forssk*) and eaten raw or cooked (**Supplementary Figure 4A**). At the time of collection, water depth was 20 cm. Water was collected from the middle of the stream and was very turbid at visual inspection.

A single sample was collected from an artificial pond with soil sediment. The pond is used for fish farming (**Supplementary Figure 4B**). A 1 L sample was taken from the shallow end of the pond. Sample was very turbid at visual inspection.

Two samples were taken from a cement container, which is used for short term holding of live fish and amphibians (**Supplementary Figure 4C**). Occasionally, the water from the pond and the cement container is used for watering gardens. A 1 L sample was collected and was slightly turbid at visual inspection.

Soil Samples

Seven soil samples were collected from a depth of no more than 5 cm using sterile spoons. Each sample consisted of 2–3 g of soil. Four of these came from four separate vegetable gardens (**Supplementary Figures 5A–D**). Three of the gardens were field plots, while one comprised of pots. One soil sample came from an ephemeral stream, where the local herb Plu Kaow grows (*Houttuynia cordata Thunb*). Villagers use this herb extensively (raw or cooked) for vegetable side dishes accompanying raw meat. The stream was void of water, but muddy at the time of collection. One sample was also gathered from river sediment. One soil sample was picked from the riverbank. Both the river and the riverbank are overgrown with morning glory (**Supplementary Figure 6**).

Blastocystis Cultures

For human and other animal fecal samples, approximately 200 mg of freshly collected feces were placed in LYSGM (Diamond, 1982) containing 10% horse serum. Water samples were left to sit for 3 h on a flat surface. Subsequently, 2–4 mL was taken from the bottom of each sample and placed in LYSGM. Soil samples were thoroughly mixed and 100 mg placed in LYSGM. Tubes were incubated at 37°C for 48–72 h and screened for *Blastocystis* using light microscopy.

Genomic DNA Extraction

Human and Other Animal Fecal Samples

In the case of human samples, DNA was extracted from feces using 200 mg. DNA from animal samples was extracted prior to the first passage of culture using 250 mL of sediment from each sample. The Qiagen DNA stool minikit (Qiagen, Hilden, Germany) was used according to manufacturer's protocol.

Water Samples

DNA was extracted from 250 mL of culture sediment using AccuPrep® Genomic DNA Extraction Kit following the manufacturer's protocol.

Soil Samples

DNA from soil was directly extracted from 200 mg of soil using PowerSoil® DNA Isolation Kit (Carlsbad, CA United States) according to manufacturer's protocol.

Blastocystis Detection

Three approaches were used to detect *Blastocystis* from human samples: microscopy following culturing in LYSGM, conventional PCR and qPCR (**Figure 2**). For the rest of the samples only microscopy and qPCR were used.

Polymerase Chain Reaction and Sequencing

The broad specificity primer pair RD3 5′-GGGATCCTGA TCCTTCCGCAGGTTCACCTAC-3′ and RD5 5′-GGAAGC TTATCTGGTTGATCCTGCCAGTA-3′ (Clark, 1997) was used for the first PCR reactions with the following conditions: initial denaturation for 3 min at 94°C, 35 cycles at 94°C for 1 min, annealing 60°C for 1 min, and extension at 72°C for 100 s, with a final elongation step at 72°C for 7 min. A 600 bp fragment of *SSU* rRNA gene region, which is also the barcode region of *Blastocystis* was amplified with a second nested PCR. The PCR reaction was carried out by using the forward BsRD5F (5′-ATCTGGTTGATCCTGCCAGT-3′) and reverse BhRDr9R (5′-GAGCTTTTTAACTGCAACAACG-3′) barcoding primers (Scicluna et al., 2006). The PCR conditions consisted of initial denaturation for 3 min at 94°C, 35 cycles at 94°C for 1 min, annealing 60°C for 1 min, and extension at 72°C for 100 s, with a final elongation step at 72°C for 10 min. Positive and negative controls were included with each batch of samples analyzed.

Quantitative Polymerase Chain Reaction

Blastocystis prevalence was assessed using qPCR to amplify a 330 bp fragment of the *SSU* rRNA gene. The qPCR reactions mixture were performed in 10 μL reaction mixture volume with 3 μL of water, 4 μL SensiFAST^{TM} SYBR No-ROX Kit (BIOLINE, United Kingdom), 0.5 μL of each forward (BL18SPPF1; 5′-AGTAGTCATACGCTCGTCTCAAA-3′) and reverse (BL18SR2PP; 5′-TCTTCGTTACCCGTTACTGC-3′) *Blastocystis*-specific primer and 2 μL of genomic DNA. The qPCR amplification conditions were as previously described (Poirier et al., 2011). Reactions were run in 96-well plates in a CFX96 Touch^{TM} Real-Time PCR Detection System (Bio-Rad, United States). Positive and negative controls were used in each qPCR run together with all samples. Each type of sample was run separately to avoid cross-contamination. For example, soil sample experiments were executed on separate plates and on separate days from water, human and animal samples.

Amplicon Purification and Sequencing

All positive PCR and qPCR products were purified using the GeneJET Gel Extraction Kit (Thermo Fisher Scientific; Wardmedic, Thailand) according to manufacturer's instructions and sequenced at U2Bio (Korea).

Cloning

Twenty-one samples showing long stretches of indistinguishable peaks were cloned, six of which were PCR products and 15 qPCR. Five samples were human, two buffalo, two pig, four chickens, four water and four soil. 1.5 μL of amplicon was used with the pGEM-T easy vector system I (Promega, Madison, WI, United States) following previously published cloning protocols (Betts et al., 2018). Up to five colonies per transformation were screened.

Phylogenetic Analysis

The chromatogram quality of raw reads was checked using the chromatogram visualization software 4Peaks. Ambiguous bases at the ends of the reads were removed. The new sequences were then used as queries to perform blast searches against the NCBI nr database. Sequences of *SSU* rRNA spanning the spectrum of *Blastocystis* diversity were downloaded and aligned using mafft v. 7.394 (Katoh and Toh, 2010). Ambiguous positions were removed using trimal v. 1.4 and gappyout option (Capella-Gutierrez et al., 2009). The final trimmed alignment consisted of 250 taxa and 1497 sites. Maximum likelihood (ML) analysis was performed in CIPRES Science Gateway ([1]Miller et al., 2010) using RAxML-HPC2 on XSEDE (Stamatakis, 2006). Bootstrap support was computed from 1,000 pseudoreplicates.

RESULTS

Human Demographic Data

A total of 45 human volunteers participated in this study (31% male, n = 14 and 69% female, n = 31), with mean age of 59.1 ± 8.5 years (median = 60).

Comparison of Microscopy and Molecular Methods in Human Stool Samples

The prevalence of *Blastocystis* in all human stool samples was observed using morphology and molecular techniques (**Table 1**). All samples were cultured in LYSGM and of these, 9% (4/45) were microscopy-positive for *Blastocystis*. Using conventional PCR, 49% (22/45) of samples were positive, while the number increased to 73% (33/45), when using qPCR. All microscopy-positive samples were also positive using molecular detection. Eleven PCR samples were false positive by Sanger sequencing (plants and fungi rather than *Blastocystis*), thus PCR positivity rate of *Blastocystis* confirmed by sequencing was 27% (12/45). One qPCR product was false positive by Sanger sequencing (Fungi; not included in the prevalence calculation). The prevalence rates

[1]https://www.phylo.org/portal2/login!input.action

TABLE 1 | Comparison of microscopy and molecular methods.

Methods	Prevalence	
	Positive	**Negative**
Morphology		
Light microscopy	4 (8.89%)	41 (91.11%)
Molecular		
Polymerase chain reaction (PCR)	12 (26.67%)	33 (73.33%)
quantitative Polymerase chain reaction (qPCR)	33 (73.33%)	12 (26.67%)

TABLE 2 | Prevalence and subtypes of *Blastocystis* in human, animal, water and soil samples.

Source	B + ve	ST1	ST2	ST3	ST4	ST5	ST6	ST7	ST10	ST23	ST26	UNK
Human	33	5	1	2	1	1	–	1	6	12	–	1
Chicken	33	–	–	–	–	–	2	31	–	–	–	–
Pig	6	–	–	–	–	–	–	6	–	–	–	–
Buffalo	4	–	–	–	–	–	–	2	–	2	–	–
Water	26	5	–	13	–	–	1	1	1	1	2	2
Soil	6	1		2	–	–	–	2	–	3	1	–
Total	**108**	**11**	**1**	**17**	**1**	**7**	**3**	**37**	**7**	**18**	**3**	**3**

reported are based only on samples that have been sequenced and are indeed verified as *Blastocystis*.

Prevalence and Diversity of *Blastocystis* in Animal and Environmental Samples

Forty-four fecal samples were collected from animals as follows: chickens (n = 34), pigs (n = 6) and buffalo (n = 4). All animal samples were cultured in LYSGM. Using microscopy, 65% (22/45) of chicken cultures were positive, while no *Blastocystis* was observed in pig and buffalo cultures. Using qPCR and subsequent sequencing, the prevalence of *Blastocystis* was 100% in chickens, pigs and buffalo. Overall, 28 samples of water and seven samples of soil were cultured and surveyed for *Blastocystis*. Two water and one soil sample were false positives for Cercozoa and bacteria and were not considered for further analysis. Prevalence using qPCR was 93% (26/28) for water and 86% (6/7) for soil. The reported prevalence rates are based solely on samples that have been sequenced and verified as *Blastocystis*.

Of the PCR and qPCR *Blastocystis* positive samples that were sequenced, 21 were cloned: Cloning yielded 62 clones, of which 17 were from human fecal samples, six from buffalo, nine from pig, 14 from chicken, nine from water and seven from soil (**Supplementary Material 2**). The following subtypes (STs) were identified: ST1, ST2, ST3, ST4, ST5, ST6, ST7, ST10, ST23, and ST26 (**Table 2**). Nine sequences could not be subtyped either because of poor quality or short length. Eight of the identified subtypes were found in humans. The dominant subtype was ST23 (12/33, 36%), followed by ST10 (6/33, 18%), ST1 (5/33, 15%), ST3 (2/33, 6%) and a single occurrence of ST2 (1/33, 3%), ST4 (1/33, 3%), ST5 (1/33, 3%), and ST7 (1/33, 3%). Chickens carried ST6 (2/33, 3%) and ST7 (31/33, 94%), pigs ST7 (6/6, 100%), and buffalo ST7 (2/4, 50%) and ST23 (2/4, 100%). Subtype 1 (5/26, 19%), ST3 (13/26, 50%), ST6 (1/26, 4%), ST7 (1/26, 4%), ST23 (1/26, 4%), and ST26 (2/26, 8%) were detected in water, whereas in the soil samples ST1 (1/6, 17%), ST3 (2/6, 33%), ST7 (2/6, 33%), ST23 (3/6, 50%), and ST26 (2/6, 33%) were found. Three humans carried both ST10 and ST23. Within subtypes, multiple genetically diverse strains were present in ST7, while ST1, ST3, ST5, and ST6 sequences were much more genetically similar (data not shown).

A detailed account of all newly generated sequences is provided in **Supplementary Material 2**. All 149 sequences generated in this study have been submitted to GenBank under accession numbers OL351649–OL351797.

Phylogenetic Analysis

All *Blastocystis* sequences grouped together with maximum bootstrap support (BS) (**Figure 3**). Subtypes 15 and 28 along with sequences from ectothermic hosts placed in the base of the tree in agreement with previous studies (Higuera et al., 2021). Subtype 5, ST12, ST13, ST14, ST24, and ST25 formed a clade sister to the clade formed by ST26, ST21, ST30, and ST32. Distinct clades of subtypes were as follows: ST6 and ST7; ST1, ST2, and ST11; ST23 and ST10; and ST4 and ST8. Newly generated sequences placed within clades consisting of known subtypes with the exception of the human origin sequence S.NO.07. Notably, the positions of the new sequences placing with ST10 and ST23 are not entirely robust, suggesting that perhaps these are new, closely related subtypes. Nonetheless, without full length sequences further conclusions cannot be drawn (this is currently under investigation).

Transmission Dynamics

Household Level

Samples were collected from a total of 39 households. In most cases, a single individual per household was sampled, with the exception of six households where all samples from all individuals were collected (**Figure 1**). Of those, five households were found positive for *Blastocystis*. In two of them, only the male occupant was positive. In the other three households, both occupants were positive, but carried different subtypes.

Farm Animal Ownership Level

Of the 39 sampled households, eight of them had animals (seven with chickens and one with buffalo). *Blastocystis* was found in six of these households and there was no subtype sharing between animal and human hosts (**Table 3**).

Environmental Level

Of the 39 sampled households, 16 were sampled for water and six for soil, all of which were positive for *Blastocystis*. There was subtype overlap between water and humans in one household (ST3).

Community Level

Out of the 108 *Blastocystis* positive samples, 33 (31%) were from humans, 43 (40%) from animals, 26 (24%) from water and 6 (6%) from soil. Subtype 2 and ST4 were identified only in humans, whereas ST26 was only found in the environmental samples (both soil and water). Subtype 7 was the most broadly distributed as it was found in humans, pig, buffalo and chicken,

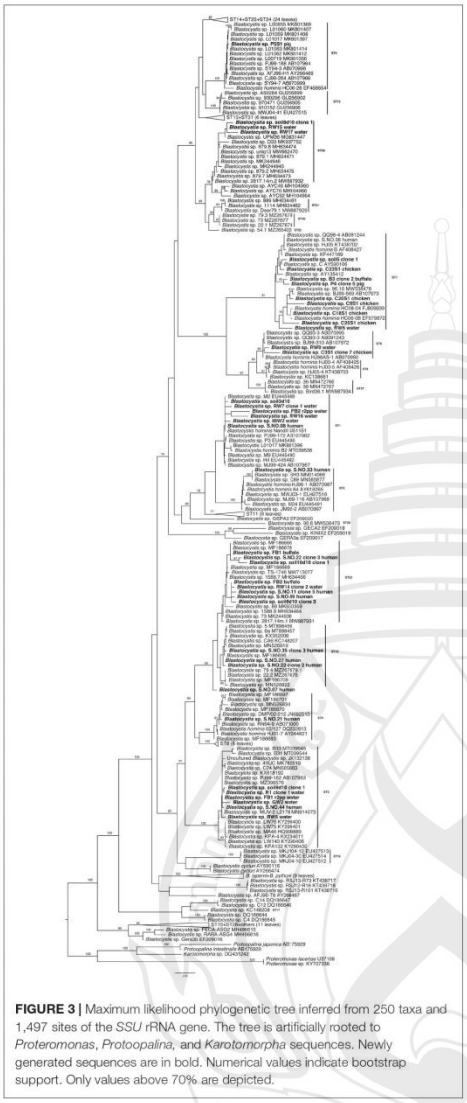

FIGURE 3 | Maximum likelihood phylogenetic tree inferred from 250 taxa and 1,497 sites of the *SSU* rRNA gene. The tree is artificially rooted to *Proteromonas*, *Protoopalina*, and *Karotomorpha* sequences. Newly generated sequences are in bold. Numerical values indicate bootstrap support. Only values above 70% are depicted.

but also in soil and water. Subtype 1, ST3, ST7, ST10, and ST23 were found in human and environmental samples. No subtype was exclusively shared by only humans and other animals. Subtype 6 was the only one shared between animals and the environment.

TABLE 3 | Prevalence of *Blastocystis* in animals and their animal-keepers.

Household	Animals	*Blastocystis* in humans	*Blastocystis* in animals
1	Chicken	Negative (*n* = 2)	ST7 (*n* = 10)
2	Chicken	ST1 (*n* = 1) ST10 (*n* = 1)	ST7 (*n* = 5)
3	Chicken	ST23 (n-1) unknown (*n* = 1)	ST7 (*n* = 5)
4	Chicken	ST23 (*n* = 1)	ST7 (*n* = 3)
5	Chicken	ST3 (*n* = 1)	ST7 (*n* = 4)
6	Chicken	Unknown (*n* = 1)	ST7 (*n* = 3)
7	Buffalo	ST4 (*n* = 1)	ST23, ST7 (*n* = 4)
8	Chicken	Negative (*n* = 1)	ST7 (*n* = 4)

DISCUSSION

The study took place in a century old isolated rural community in northern Thailand comprising approximately 500 people. Inhabitants live in very close proximity to their animals, primarily chickens and secondarily buffalo and pigs. Part of the community's water supply comes from the river that runs through it. The river also provides a major food source for the villagers, as fish constitutes the primary protein source of the community, along with vegetables (which also grow inside the river and the river bank) and locally farmed sticky rice. The increased influence of westernized diet noted in urban centers of Thailand has a minor impact in this community. Collectively, the small population, distance from urban centers, unique gastronomy (minimal effect from westernization) and the general lifestyle make this particular community ideal for local One Health approaches. Herein, we used *Blastocystis*, a microbial eukaryote of controversial pathogenicity, to obtain a comprehensive view of its transmission dynamics.

Blastocystis is the most frequently encountered intestinal protist of metazoans with most studies focusing on either its prevalence in humans, other animals and/or the environment. Nonetheless, only very few investigations explore the organism's transmission dynamics using a tripartite approach, whereby all of the aforementioned factors are considered collectively. In order to understand the role of this organism in health and disease it is essential to determine its occurrence simultaneously in human and non-human hosts and environments.

In humans, the prevalence of *Blastocystis* has been frequently reported in those with and without gastrointestinal symptoms (Dogruman-Al et al., 2009b; Scanlan et al., 2014; Yowang et al., 2018; Kataki et al., 2019; Lhotská et al., 2020; Padukone et al., 2020). Overall prevalence of *Blastocystis* might vary due to sampling population, region and detection method (Stensvold et al., 2009b; Tan et al., 2010; Alfellani et al., 2013a; Anuar et al., 2013; Clark et al., 2013). Herein, the prevalence of *Blastocystis* in asymptomatic human hosts was 73%, in asymptomatic non-human hosts 100% and in environmental samples 91%. We used microscopy and molecular methods to determine presence of *Blastocystis*. The most sensitive detection method was

qPCR matching previous studies (Poirier et al., 2011; Stensvold and Nielsen, 2012; Stensvold et al., 2012). After sequencing all positive samples, a broad diversity of subtypes (STs) was detected: ST1, ST2, ST3, ST4, ST5, ST6, ST7, ST10, ST23, and a potential new subtype. Subtype 10 was detected in six human volunteers. The subtype has been previously found in two Senegalese children (Khaled et al., 2020), but it is a typical cattle subtype (Cian et al., 2017; Zhu et al., 2017; Masuda et al., 2018; Wang et al., 2018). To our great surprise, we found ST23 in 12 human samples making it the dominant subtype in this host. So far, ST23 has only been identified in ruminants. The occurrence of ST10 and ST23 in several adults in an Asian country raises questions regarding the host range and transmission dynamics of *Blastocystis* subtypes.

The following transmission routes have been widely discussed for *Blastocystis*: human-to-human, animal-to-human and environment-to-human. The former mode of transmission has been speculated to occur via the fecal-oral route much like other common gastrointestinal parasites. Herein, investigation of individuals within households showed no subtype sharing and there was even an instance of co-habiting individuals, whereby one was *Blastocystis* positive and another negative. This finding matches previous recent reports derived from family units elsewhere (Scanlan et al., 2016; Lhotská et al., 2020).

We also aimed to look at the animal-to-human transmission route. Previous studies have suggested that specific subtypes are zoonotic (Parkar et al., 2010; Alfellani et al., 2013b; Wang et al., 2014). For instance, ST5 has been proposed as potentially zoonotic from pigs (Yan et al., 2007; Wang et al., 2014) and *Blastocystis* ST6 and ST7 from poultry (Ramírez et al., 2014; Cian et al., 2017; Greige et al., 2018; Udonsom et al., 2018). Subtype 1, ST7, ST10, and ST23 were found in both human and animal hosts in the studied area giving the impression of zoonotic transmission. However, when looking at a fine-scale level there was no sharing of subtypes between animals and their respective owners. Collective consideration of the evidence points toward the source of *Blastocystis* in this specific community being elsewhere.

This prompted us to look at the two most commonly encountered environmental sources in the community: water and soil. Water contamination has been speculated as a risk factor to acquire *Blastocystis*. However, only few studies have looked at presence of *Blastocystis* in both water and humans that use it and even fewer have employed subtyping to examine overlap between the two (Leelayoova et al., 2008; Angelici et al., 2018; Pawestri et al., 2021). *Blastocystis* has been detected in drinking water (Leelayoova et al., 2008), tap water (Eroglu and Koltas, 2010), rain water tanks (Noradilah et al., 2017; Waters et al., 2019), bodies of freshwater (Ithoi et al., 2011; Khalifa et al., 2014), drinking water treatment facilities (Richard et al., 2016) and waste water (Suresh et al., 2005; Banaticla and Rivera, 2011; Stensvold et al., 2020). Herein, *Blastocystis* ST1 and ST3 were detected in community supply water, while ST1, ST3, ST5, ST6, ST7, ST10, ST23, and ST26 were found in rain collection vessels. Both these sources comprise the drinking water of this community. The rain collection vessels contain water that is filtered for large debris, but the water is untreated and is consumed unboiled (Li et al., 2007; Leelayoova et al., 2008; Anuar et al., 2013; Wongthamarin et al., 2018; Waters et al., 2019). The community supply water is filtered and occasionally treated. Given the exposed nature of the community water, various wildlife animal hosts harboring a range of subtypes (known and unknown) can easily access it. Thus, presence of the organism in these two sources could be due to a combination of factors including contamination by animal droppings and/or substandard management (i.e., filtration and chlorine usage). Water in the vessels is also used to wash vegetables and tubers hence transfer of cysts of a variety of subtypes could occur this way. Indeed *Blastocystis* has been previously found in vegetables (Al Nahhas and Aboualchamat, 2020; Li et al., 2020). Through fine-scale analysis we identified a case of ST3 in humans overlapping with the subtypes found in their rain collection vessels. Presence of *Blastocystis* in an environment, where there is continuous circulation of oxygen supported recently raised hypotheses that this previously considered strictly anaerobic organism tolerates oxygen (Tsaousis et al., 2012, 2018). Thus, future studies should aim toward investigating additional environments including extreme habitats for the presence of *Blastocystis*.

To that end, we broadened our approach and also explored occurrence of *Blastocystis* in soil. Most collected soil samples were positive for the organism, while ST1, ST3, ST7, ST23, and ST26 were identified. To our knowledge this is the first report of this protist being recorded in natural soil. The presence of *Blastocystis* in the soil could be due to extensive use of animal excrement and intestinal contents (especially from fish), which are typically utilized as garden fertilizer in the community. Nonetheless, while sampling, care was taken to collect from gardens that had not been recently fertilized. Moreover, wildlife hosts roaming the community could also shed *Blastocystis*. This finding suggests a new route of transmission that has been previously overlooked. In that vein, we propose that soil should not only be checked for presence of the organism in future studies, but that it should also be included along with water as a transmission route in the life cycle of *Blastocystis* (**Figure 4**).

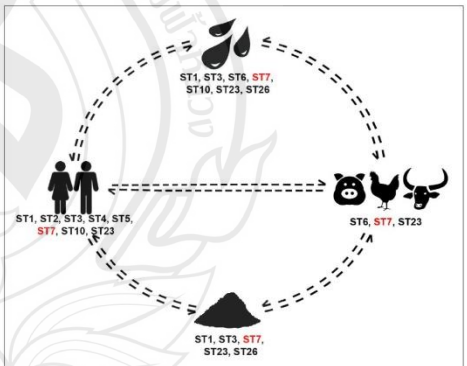

FIGURE 4 | *Blastocystis* subtype cycling in the rural community studied herein. Subtypes present in all sources are in red font.

Comparison of sequences found in different hosts and environments indicated that highly similar strains of ST1 and ST3 are circulating in the community. This suggests a shared *Blastocystis* transmission cycle among humans, animals and the environment for these subtypes. In contrast, ST7 showed an extraordinary amount of diversity with multiple strains distributed within and between hosts and the environment. This indicates that the full extent of ST7 genetic diversity and host range in the community has yet to be captured. Nonetheless a cluster of highly similar strains was found in chicken, pig and buffalo suggesting transmission among the three hosts.

This study has revealed no clear patterns of direct transmission between human-to-human or animal-to-human in this community (**Figure 4**). Instead, it points out to the existence of multiple independent routes of transmission. Previous efforts investigating *Blastocystis* sources of transmission have been geared toward dissecting dipartite relationships (i.e., animal-to-human or environment-to-human). Results from these studies have enhanced our understanding of the organism and its epidemiology. Nonetheless, they frequently only provide pieces of the overall picture, which remains fragmentary. Here, we have provided a step forward toward integrating a One Health approach to *Blastocystis* by considering both living and non-living sources. In this community, environmental sources comprise the reservoir of *Blastocystis* supplying a multitude of subtypes that circulate in both human and non-human hosts. Our study is pioneer in that we investigated a rural area, while taking into account the community structure and environmental factors toward understanding *Blastocystis* circulation.

Limited sample size does pose a limitation in our study. Specifically, the sample size was low, in particular samples from various animal hosts including stray animals and wildlife. Thus transmission cycles between and within hosts and the environment cannot be precisely deduced at this time.

Moving forward, additional communities both rural and urban should be explored under the One Health umbrella to determine whether similar patterns occur. Using the same approach in a temporal context, future studies should also investigate, whether *Blastocystis* and its various subtypes are true colonizers or passengers. Finally, supplementing One Health-based studies with culturomics and microbiome (pathogenic and non-pathogenic residents of the gut) and metabolome investigations will contribute significantly in uncovering the true roles of *Blastocystis* in gut health and disease.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/ **Supplementary Material**.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Ethics Committee of Mae Fah Luang University. The patients/participants provided their written informed consent to participate in this study. The animal study was reviewed and approved by the Ethics Committee of Mae Fah Luang University. Written informed consent for participation was not obtained from the owners because only fecal samples were obtained, there was no invasive procedure involved.

AUTHOR CONTRIBUTIONS

VJ: fieldwork, methodology, conceptualization, investigation, and draft writing. SM: methodology. SP: resources and methodology. AT: supervision, validation, and final draft. EG: funding acquisition, project administration, supervision, validation, data curation, and final draft. All authors have read and approved the submitted version of the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2021.746340/full#supplementary-material

Supplementary Material 1 | Includes **Supplementary Figures 1–6**, which are images from the collection sites.

Supplementary Material 2 | Includes a detailed account of all newly generated sequences.

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Research paper

Blastocystis in tap water of a community in northern Thailand

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ABSTRACT

Blastocystis is the most common protist in the gut of humans and other animals having global distribution. Occasionally, this organism has also been reported in the environment. Transmission to humans occurs via the fecal-oral route, while water also comprises a transmission route. *Blastocystis* has been commonly found in rivers, lakes, and wells. Nonetheless, there is limited data about the prevalence and genetic diversity of *Blastocystis* in tap water. The main aim of this study was to examine the presence of *Blastocystis* subtypes in tap water (n=20) in a community in northern Thailand. Molecular characterization using the small subunit ribosomal RNA was used to screen for *Blastocystis* and identify the diversity of subtypes in samples. The overall prevalence was 30% with only subtype three (ST3) encountered in the tap water. These results indicate that tap water has a potential role in the transmission of this subtype in the studied community. Further investigations should focus on expanding sampling to include additional housing complexes and screening for *Blastocystis* in humans who are exposed to this water.

1. Introduction

Blastocystis is one of the most common protists in the intestinal tract of humans and other animals with worldwide distribution [1–3]. *Blastocystis* has four morphological forms, namely vacuolar, granular, amoeboid, and cyst [4,5]. The advent of molecular methods over the past several years, has revealed that the organism has an astounding genetic diversity. Based on the small subunit ribosomal RNA (SSU rRNA) gene, a total of 28 subtypes (STs) of *Blastocystis* have been identified: ST1-ST17, ST21, ST23-ST32 [6–13]. Subtypes ST1-ST17 have been accepted for a long time, while the rest were proposed more recently. So far, ST1-ST10, ST12, ST14, ST16 and ST23 have been found in humans, with ST1-ST4 being the most frequently reported [14–17]. Nonetheless, these subtypes, have also been found in other hosts, indicating the non-host specific nature of *Blastocystis*. The exception to this is ST9 which so far has only been found in humans.

Blastocystis transmission occurs via the fecal-oral route and it has been proposed that the cyst is the transmissible form [18]. Several avenues of transmission have been explored including human-to-human and zoonotic, or via contaminated food and water [4,17,19,20] Zoonotic transmission has been considered as commonly occurring. For example, *Blastocystis* ST5 has been detected in pigs and pig handlers [21,22], ST7 has been found in avian hosts and people living within the same area, while ST8 has been reported in human and non-human primates [6,7,23]. In recent years, water sources have been implicated in the transmission cycle of *Blastocystis*. Presence of the organism has been associated with humans who consumed untreated water as well as with those who had no access to piped water supply [24,25]. Several studies have investigated the occurrence of *Blastocystis* in water environments, including rivers, lakes, wells and rain storage containers [17,24–35].

Blastocystis in tap water has been reported only occasionally [36–38]. In this pilot study we investigated presence of *Blastocystis* in tap water in a semi urban area of northern Thailand. Our objective was to determine whether water comprises a source of acquiring *Blastocystis* in this specific community.

2. Methods

2.1. Samples collection, culturing and microscopy

Tap water samples were collected from 20 randomly selected housing complexes in Chiang Rai Province, Thailand (Fig. 1). These

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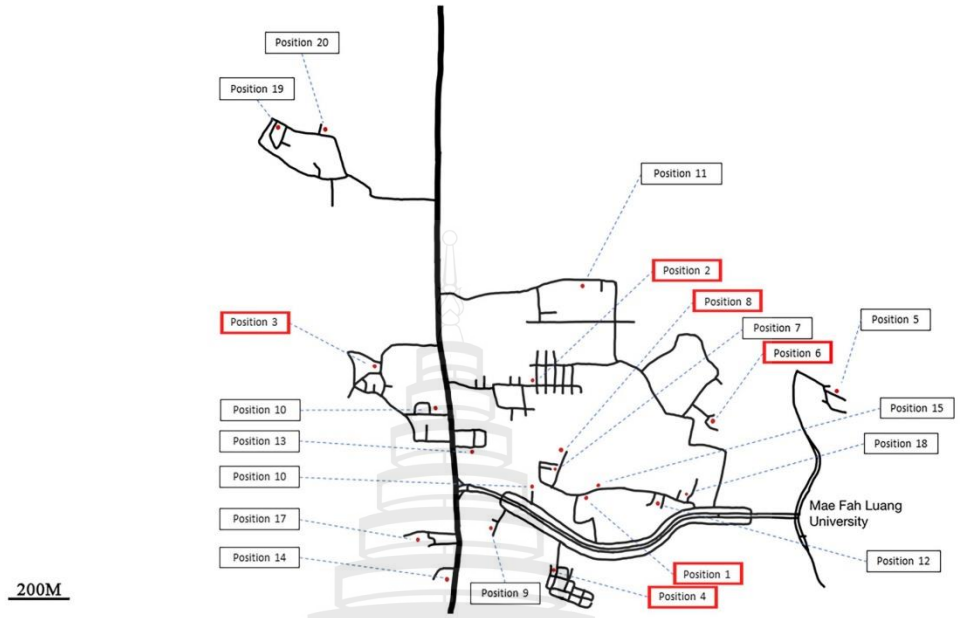

Fig. 1. detail of area of collection in this study. Red dots indicate housing complexes from which samples were collected. Red squares indicate samples positive for *Blastocystis*. *(For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)*

complexes house mostly university students. Samples were collected during the wet season between July and August 2021. The source of tap water for 19 housing complexes was verified as coming from groundwater. In 13 of these, water is cleaned by filtration, while in three the tap water is chlorinated. The water in three of the complexes in neither filtered nor chlorinated. The tap water of one of the housing complexes comes from a water reservoir storing river water and is cleaned by filtration (Supplementary Fig. 1). Tap water was stored in polyvinyl (n = 16), stainless steel (n = 3), or metal (n = 1) containers. Tap water in this study is used for bathing, washing food and clothes and watering plants. Tap water (2–5 ml) was placed in LYSGM as previously described [17] and incubated at 37 °C. A tube containing only LYSGM media was used as control. All cultures were screened for *Blastocystis* using light microscopy.

2.2. Genomic DNA extraction

Total genomic DNA was extracted using 200 mg of sediment from culture and LYSGM control using high molecular weight extraction protocol [39]. Briefly, 1 mL of each culture was homogenized with cell lysis buffer and incubated at 75 °C. Afterwards, 100 mg/mL of lysozyme was added, and the mixture was incubated at 37 °C overnight. Subsequently, 10% SDS, 25 mg/mL of proteinase K, and 20 mg/mL of RNase were added and incubated at 55 °C for 30 mins. Phenol/chloroform/isoamyl alcohol (25:24:1) was added and the tubes were centrifuged at 13,000 x*g* for 5 min. Residual contamination of phenol/chloroform was removed by adding chloroform/isoamyl alcohol (24:1) and centrifugation at 13,000 x*g* for 5 min. The aqueous phase was kept, and the rest discarded. The DNA was precipitated with 3 M sodium-acetate and isopropanol by centrifugation at 13,000 x*g* for 5 min. The DNA pellet was washed with 70% ethanol, air-dried, and re-suspended in TE buffer.

2.3. Quantitative polymerase chain reaction (qPCR)

A fragment of the SSU rRNA gene of *Blastocystis* was amplified using quantitative polymerase chain reaction (qPCR) analysis. This method of detection was chosen due to its sensitivity and low rate of false positives in our previous studies [17]. The SensiFAST™ SYBR No-ROX Mix (Bioline company, U. S. A) was used in the qPCR reaction. The PPF1 (5'-AGTAGTCATACGCTCGTC TCAAA-3) and R2PP (5'-TCTTCGTTACCCGTTACTGC-3′) primers were used [40]. The expected product size was 330 bp. The qPCR conditions were as follows: initial denaturation at 95 °C for 5 min followed by 49 cycles of denaturation at 95 °C for 5 s, annealing at 68 °C for 10 s, and 72 °C for 15 s. Two negative controls were used; one containing all reagents except template and one containing the DNA extracted from the LYSGM media control. Reactions were run on a Bio-Rad/CFX96 Touch Real-Time PCR Detection System. The amplicon products positive for *Blastocystis* were purified using GeneJET Gel Extraction Kit (Thermo Scientific; Wardmedic, Thailand) according to the manufacturer's instructions and sequenced (Bionics Company, Korea).

2.4. Phylogenetic analysis

The chromatogram quality of all reads was checked using the chromatogram visualization software 4Peaks. Ambiguous bases at the ends of the reads (5′ and 3′) were removed. The newly obtained sequences were used as queries to perform BLAST searches against the GenBank database to ensure they were not contaminants or chimeras. Subtype Sequence Typing (MLST) (available at https://pubmlst.org/blastocystis

Table 1
Water containers that were sampled in this study and their corresponding purification systems.

Position	Container type	Chlorination	Filtration	Detection of *Blastocystis*
1	Polyvinyl	−	−	+
2	Polyvinyl	+	−	+
3	Polyvinyl	−	+	+
4	Polyvinyl	+	−	+
5	Metal	−	+	−
6	Polyvinyl	−	+	+
7	Polyvinyl	−	+	−
8	Polyvinyl	+	−	+
9	Polyvinyl	−	+	−
10	Polyvinyl	−	+	−
11	Stainless steel	−	+	−
12	Polyvinyl	−	−	−
13	Polyvinyl	−	+	−
14	Stainless steel	−	+	−
15	Stainless steel	−	+	−
16	Polyvinyl	−	+	−
17	Polyvinyl	−	+	−
18	Polyvinyl	−	−	−
19	Polyvinyl	−	+	−
20	Polyvinyl	−	+	−

/) was also used to identify subtype and the corresponding allele. The SSU rRNA gene sequences of all currently accepted *Blastocystis* subtypes were used to construct the dataset. In total, 119 sequences were aligned using the software MAFFT v.7 [41] and ambiguous regions were removed using trimAL v.1.4 [42]. After trimming the alignment contained 1336 sites. A maximum likelihood (ML) phylogenetic tree was built using RAxML v.8 [43] the general time reversible + Γ model of sequence evolution and 1000 bootstrap replicates.

3. Results

Sediments from three cultures were positive for *Blastocystis* by light microscopic examination (results not shown). Ten of the 20 tap water samples (50%) were qPCR positive. However, after sequencing, only six of the ten were identified as *Blastocystis*. The other four were falsely positive and represented sequences that cannot be identified in the database. Thus the overall occurrence of *Blastocystis* in tap water was 30%. All six *Blastocystis* positive sequences were identified from tap water coming from groundwater. Of these six, one sample was neither chlorinated nor filtered, three were chlorinated but not filtered, and two were filtered, but not chlorinated (Table 1). Water from all six samples was stored in polyvinyl containers after filtration/chlorination.

In the phylogenetic analysis, all six newly derived sequences claded with ST3 (Fig. 2). However, the possibility of other subtypes being present in the water and not established in culture cannot be excluded. All subtypes were monophyletic. In agreement with previous studies, *Blastocystis* isolates from ectothermic hosts along with ST15, and ST28 placed at a basal position. The closest allele match for all six sequences was allele 34. Sequence SW06 was identical to that of allele 34 in the MLST database, sequences SW01, SW02 and SW04 differed by a single nucleotide, while sequences SW08 and SW03 had two and five differences, respectively. The sequences generated in this study have been submitted to GenBank under accession numbers OP000917-OP000922.

4. Discussion

In this pilot study we surveyed housing complexes for the presence of *Blastocystis* in tap water following previously established protocols [37]. Presence of *Blastocystis* in tap water has not been frequently explored, and in Thailand such surveys are sparse. *Blastocystis* was identified in 30% of the sampled tap water.

Though *Blastocystis* has been reported in a variety of water environments, including treated potable rain water, water tanks, ponds, canal water and wastewater, subtype information is not always available [27,29,30,34]. Nonetheless, a variety of subtypes have been identified in water. Subtype 1 has been reported in drinking water, non-potable water and tap water [36,37], while ST1, ST2, ST6 and ST8 have been found in wastewater treatment plants [26,32]. A variety of *Blastocystis* subtypes (ST1, ST3, ST6, ST7, ST10, ST23 and ST26) were found in water bodies and containers in a rural community in northern Thailand [17]. Worldwide, ST1–4 have been identified in river samples [28,33,35,38]. The organism has also been found in unboiled drinking water, rivers, tap water as well as natural water bodies and rain water collection tanks [17,24–38]. Subtype 1 and ST3 are the most commonly encountered in water sources, while ST2, ST4, ST6–8, ST10, ST23 and ST26 have occurred only sporadically [17,26,28,31–33,35–38]. Herein, all samples positive for *Blastocystis* belonged to ST3. No other subtypes were detected. This is in contrast to the study by Jinatham et al. (2021) [17], who detected several subtypes in water. One explanation for this could be that the media used might encourage growth of some subtypes, but not others. Both studies used the same media (LYSGM) so one can at least conclude that several subtypes can grow in this medium. Our study suggests *Blastocystis* ST3, is circulating in the water of the sampled community and poses a possible risk factor of *Blastocystis* transmission. Thus, more studies examining occurrence of *Blastocystis* from various bodies of water and geographical regions are needed to understand distribution of this organism in the environment.

Contaminated water has been implicated as a source of transmission for this organism [17,36,38]. Nonetheless, only a few studies have demonstrated that water is a route of *Blastocystis* transmission by examining both water and human hosts. For instance, *Blastocystis* ST1 and ST3 were positively associated with untreated water and drinking unboiled water, respectively [31,36]. Jinatham et al. (2021) [17] identified *Blastocystis* ST3 in humans and in water from their rain collection vessels. Previous studies have shown high prevalence of *Blastocystis* ST1 and ST3 in people who consumed untreated or contaminated water [31,37].

To our knowledge, not many studies have looked for *Blastocystis* in tap water directly. The findings herein match those of Eroglu and Koltas (2010) [37] in Turkey, who also found *Blastocystis* in tap water. Leelayoova et al. (2008) [36] reported *Blastocystis* ST1 in drinking water consumed by elementary school children in a rural Thai community. In this study, water in which *Blastocystis* was identified was either chlorinated or filtered, but not both. The presence of *Blastocystis* in tap water that is filtered was surprising. One possibility is that the organism is present in the groundwater supply. Animal feces contaminated with *Blastocystis* might be transferred into the groundwater with soil and/or sand. Alternatively, *Blastocystis* could have entered the storage container after filtration since water can sit in the container for several days. A potential alternative possibility is that the organism cannot only escape conventional water filtration, but is also resistant to chlorine. The frequency of filter changing and chlorination standards for each residence are unknown. *Blastocystis* resistance to chlorine had been previously explored [36,44]. Moreover, it has been proposed that *Blastocystis* can also survive in water for long periods of time [45].

The present study has several limitations including a small sample size. Future studies should increase sample size in terms of both number of housing complexes and residences within the complexes. Samples should also be taken from the storage containers directly as well as the groundwater and compared with those found in the taps. Finally, it would be interesting to see whether the human occupants of the *Blastocystis* positive dorms are also positive for the organism. Our study is one of the few that explore *Blastocystis* occurrence in tap water. Further investigations under the umbrella of one health should be undertaken in the future to understand the distribution of the organism in the environment and the water supply and consequently its transmission dynamics.

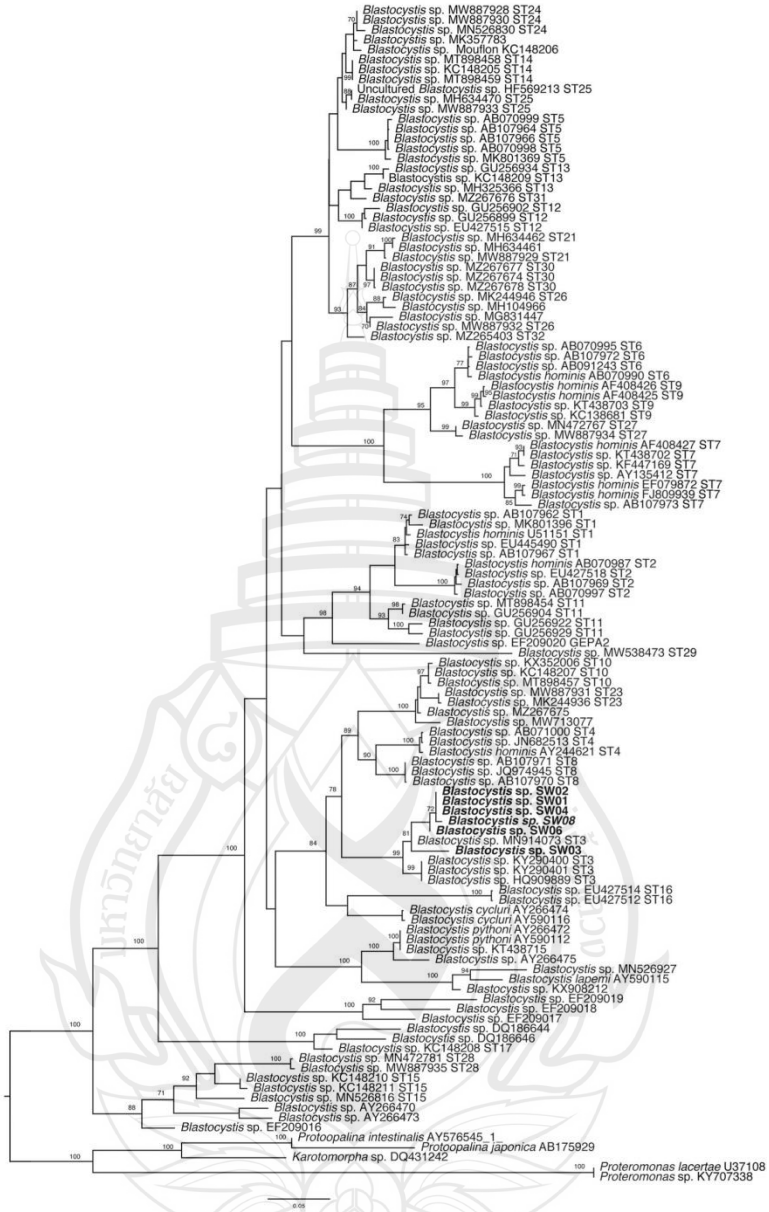

Fig. 2. Maximum likelihood phylogenetic tree inferred from 1336 sites of the SSU rRNA gene and 119 taxa. Newly generated sequences are depicted in bold font. Numerical values at the nodes indicate bootstrap support. Values below 70% are not shown.

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Declaration of Competing Interest

The authors declare that they have no conflict of interest.

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PEER REWIEW PUBLICATIONS

Jinatham, V., Nonebudsri, C., Wandee, T., Popluechai, S., Tsaousis, A. D., & Gentekaki, E. (2022). *Blastocystis* in tap water of a community in northern Thailand. *Parasitology International*. *91***,** 102624**.**

Betts, E. L., Newton, J. M., Thompson, G. S., Sarzhanov, F., Jinatham, V., Kim, M. J., . . . Tsaousis, A. D. 2021. Metabolic fluctuations in the human stool obtained from *Blastocystis* carriers and non-carriers. *Metabolites*, *11*(12), 883.

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Jinatham, V., Popluechai, S., Clark, C. G., & Gentekaki, E. (2019). *Entamoeba chiangraiensis* n. sp. (Amoebozoa: Entamoebidae) isolated from the gut of Asian swamp eel (*Monopterus albus*) in northern Thailand. *Parasitology*, *146*(14): 1719-1724. https://doi.org/ 10.1017/S0031182019000775

Jinatham, V., Kullawong, N., Kespechara, K., Gentekaki, E., & Popluechai, S. (2018). Comparison of gut microbiota between lean and obese adult Thai individuals. *Korean Journal of Microbiology and Biotechnology*, *46*(3), 277-28.

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BOOK CHAPTERS

Tsaousis, A. D., Betts, E. L., McCain, A., Newton, J. M., Jinatham, V., & Gentekaki, E. (2020). *Exploring the biology and evolution of Blastocystis and its role in the microbiome*. In: Eukaryome impact on human intestine homeostasis and mucosal immunology (pp.61-74). Springer. ISBN 978-3-030-44825-7.

ORAL PRESENTATIONS AND POSTERS

#presenter

Jinatham, V., Maxamhud, S., Popluechai, S., Tsaousis, A. D.,[#] & Gentekaki, E. (2021). *Blastocystis* One Health approach in a rural community of northern Thailand: Prevalence, subtypes and novel transmission routes. [Paper presentation]. *Microbiology Society*, Ireland.

Jinatham, V[#],. Popluechai, S., Tsaousis, A. D., & Gentekaki, E. (2021). *Blastocystis* transmission dynamics in a rural community of northern Thailand. [Conference presentation]. 3rd International *Blastocystis* Conference, UK: 4th of June 2021, online

Salomaki, E[#],. Wisniewska, M., Pelesz, A., Taborsky, P., Mazancova, M., Jinatham, V., Gentekaki, E., Cepicka, I., & Kolisko, M. (2019). Phylogenomics and comparative transcriptomics of secondarily free-living diplomonads. [Poster presentation]. *European Congress of Protistology*, Italy

Yowang, A., Jinatham, V., McCain, A., Tsaousis, A. D., Kullawong, N., Popluechai, S., & Gentekaki, E[#]. (2019). Diversity of eukaryotic gut microbiota of northern Thai populations. [Poster presentation]. *Microbiology Society*, *1*(1A), 557.

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Jinatham, V[#]., Yowang, A., McCain, A., Kullawong, N., Tsaousis, A. D., Popluechai, S., & Gentekaki, E. (2018). Diversity, prevalence and distribution of *Blastocystis* in asymptomatic volunteers living in Thailand [Poster presentation], *International Society for Evolutionary Protistology*. Cyprus

Jinatham V#., Kullawong, N., Kespechara, K., & Popluechai. S. (2015). Quantification of *Lactobacillus* spp. from fecal samples of Thai overweight and obese volunteers [Poster presentation]. *The 8th The Asian Conference for Lactic Acid Bacteria* (ACLAB). Bangkok, Thailand

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