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Title: High diversity of Blastocystis subtypes isolated from asymptomatic adults living in Chiang Rai, Thailand

Article Type: Research paper

Keywords: Blastocystis; genetic diversity; prevalence; subtyping; Thailand

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Abstract: Blastocystis is a common and broadly distributed microbial eukaryote inhabiting the gut of humans and other animals. The genetic diversity of Blastocystis is extremely high comprising no less than 17 subtypes in mammals and birds. Nonetheless, little is known about the prevalence and distribution of Blastocystis subtypes colonising humans in Thailand. Molecular surveys of Blastocystis remain extremely limited and usually focus on the central, urban part of the country. To address this knowledge gap, we collected stool samples from a population of Thai adults (n=178) residing in Chiang Rai Province. The barcoding region of the small subunit ribosomal RNA was employed to screen for Blastocystis and identify the subtype. Forty-one stool samples (23%) were identified as Blastocystis positive. Six of the nine subtypes that colonise humans were detected with subtype (ST) three being the most common (68%), followed by ST1 (17%) and ST7 (7%). Comparison of subtype prevalence across Thailand using all publicly available sequences showed that subtype distribution differs among geographic regions in the country. ST1 was most commonly encountered in the central region of Thailand, while ST3 dominated in the more rural north and northeast regions. ST2 was absent in the northeast, while ST7 was not found in the center. Thus, this study shows that ST prevalence and distribution differs not only among countries, but also among geographic regions within a country. Potential explanations for these observations are discussed herewith.

April 8, 2018

Editorial Board,
Infection Genetics and Evolution

Dear Editors,

Here we submit a revised version of our manuscript entitled: **"High diversity of *Blastocystis* subtypes isolated from asymptomatic adults living in Chiang Rai, Thailand"**.

We have addressed all the comments from the reviewers and hope the manuscript is now suitable for publication.

The manuscript contains only original data that have not been submitted or published elsewhere. We look forward to hearing from you regarding our revised manuscript.

Sincerely,

Eleni Gentekaki, Ph.D.

We thank the reviewers for the positive comments on our manuscript. Below, we address the reviewers' points. Replies are in bold lettering.

Reviewer #1

I think the title is not fit to this manuscript, because the new data obtained in this study was limited from northern region of Thailand.

We have modified the title as follows: High diversity of *Blastocystis* subtypes isolated from asymptomatic adults living in Chiang Rai, Thailand

Line 51-:
Description in this sentence is incorrect, because there are many Blastocystis organisms had been identified in addition to 17 kinds of the STs.

We have added the following sentence to account for the lineages composed of reptilian, amphibian and insect sequences and which have not been assigned to subtypes. Lines 54-56: Isolates from avian and mammalian hosts are currently classified into 17 subtypes (STs) based on the divergence of the small subunit ribosomal RNA (SSU rRNA), though new STs might be arising (Alfellani *et al.*, 2013c; Betts *et al.*, 2018;). *Blastocystis* isolated from reptilian, amphibian and insect hosts are distinct and are not assigned into subtypes.

Lines 56-:
I think that ST9 is only isolated from humans.

We have added the following sentence in lines 59-60: The exception is ST9, which has yet to be found in a non-human host.

Line 153-:
I am confusing " the alignment contained 1,379 sites", because only barcode-region (ca. 600bp) was amplified by the nested-PCR for sequencing in this study.

Indeed, even though we amplified the barcode region, our alignment contained many full-length sequences (~1800bp). Thus, after trimming, the remaining sites were more than 600bp. This is standard practice in phylogenetics, whereby missing data is the norm. By doing this we improve resolution of the phylogenetic tree and strengthen support at the nodes. The topology of the tree remains unchanged. Many investigators do the same. For instance, Alfellani et al., 2013. Genetic diversity of Blastocystis in Livestock and zoo animals, also amplified the barcoding region, but their alignment contained 1,462 sites.

Discussion
I feel the discussion is too long for the results, pleases concise the discussion. Several parts may be deleted where doesn't match the purpose of this study (lines 83-).

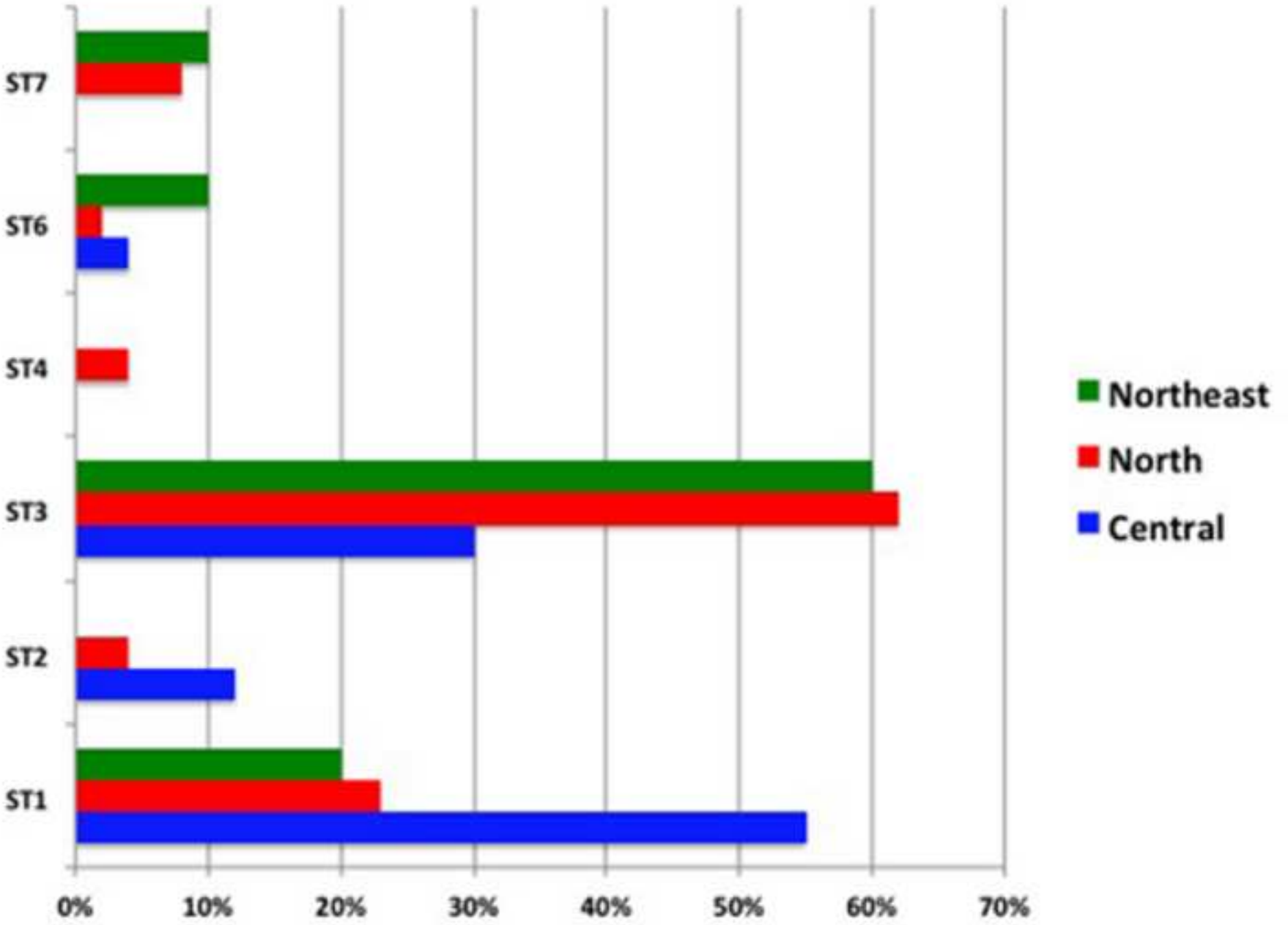
Our discussion consists of four parts: 1) asymptomatic carriage of Blastocystis, 2) comparison of prevalence rates to other studies in Thailand, 3) subtypes detected in this study, and 4) subtype distribution in Thailand. We have shortened the discussion in parts three and four.

Reviewer #2

My only comment is that it is a small study sample number for the Chiang Rai study population and all are asymptomatic. This makes it difficult to know the true prevalence of Blastocystis in this region. Only using asymptomatic people means that you might be missing a large number of positives and potential different STs which may come from symptomatic people. This limitation should be stated in the discussion.

We agree with the reviewer's comment. The purpose of this pilot study was to examine which subtypes circulate in asymptomatic individuals. To that end, we have changed the title to reflect our study site and sampled population and we have also emphasized this in the discussion (Lines: 204-205, 270-271, 278-279).

Graphical Abstracts



KEY FINDINGS

- First study of *Blastocystis* prevalence and subtyping in Chiang Rai Province, Thailand
- Six of the nine subtypes known to colonize humans were found
- ST3 is the dominant ST followed by ST1 and ST7
- Distribution of *Blastocystis* subtypes among geographic regions of Thailand differs

*Manuscript
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High diversity of *Blastocystis* subtypes isolated from asymptomatic adults living in Chiang Rai, Thailand

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Running title: *Blastocystis* diversity and subtyping in Thai adults

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Abstract

Blastocystis is a common and broadly distributed microbial eukaryote inhabiting the gut of humans and other animals. The genetic diversity of *Blastocystis* is extremely high comprising no less than 17 subtypes in mammals and birds. Nonetheless, little is known about the prevalence and distribution of *Blastocystis* subtypes colonising humans in Thailand. Molecular surveys of *Blastocystis* remain extremely limited and usually focus on the central, urban part of the country. To address this knowledge gap, we collected stool samples from a population of Thai adults (n=178) residing in Chiang Rai Province. The barcoding region of the small subunit ribosomal RNA was employed to screen for *Blastocystis* and identify the subtype. Forty-one stool samples (23%) were identified as *Blastocystis* positive. Six of the nine subtypes that colonise humans were detected with subtype (ST) three being the most common (68%), followed by ST1 (17%) and ST7 (7%). Comparison of subtype prevalence across Thailand using all publicly available sequences showed that subtype distribution differs among geographic regions in the country. ST1 was most commonly encountered in the central region of Thailand, while ST3 dominated in the more rural north and northeast regions. ST2 was absent in the northeast, while ST7 was not found in the center. Thus, this study shows that ST prevalence and distribution differs not only among countries, but also among geographic regions within a country. Potential explanations for these observations are discussed herewith.

Keywords: *Blastocystis*; genetic diversity; prevalence; subtyping; Thailand

1. Introduction

Blastocystis is the most commonly found protist in the gastrointestinal tract of humans and other animals (Roberts *et al.,* 2013; Tan, 2008). Though *Blastocystis* exhibits morphological stasis, it has a high degree of genetic heterogeneity. Isolates from avian and mammalian hosts are currently classified into 17 subtypes (STs) based on the divergence of the small subunit ribosomal RNA (SSU rRNA), though new STs might be arising (Alfellani *et al.,* 2013c; Betts *et al.,* 2018;). *Blastocystis* isolated from reptilian, amphibian and insect hosts are distinct and are not assigned into subtypes. So far, only ST1-ST9 have been found in humans with ST1-ST4 being the most common colonisers (Alfellani *et al.,* 2013b). However, these STs have also been detected in a variety of non-human hosts, suggesting that *Blastocystis* has low host specificity (Stensvold and Clark 2016). The exception is ST9, which has yet to be found in a non-human host. The rest of the STs have so far been identified in both domesticated and wildlife animal hosts (Alfellani *et al.,* 2013a).

Lately, considerable research effort has gone into deciphering potential roles of *Blastocystis* in intestinal disease. For instance, an association between presence of *Blastocystis* and irritable bowel syndrome has been discussed frequently, though a definitive causal link has yet to be postulated (Jimenez-Gonzalez *et al.,* 2012; Khademvatan *et al.,* 2017; Poirer *et al.,* 2012; Yakoob *et al.,* 2010). Nonetheless, emerging data have shown presence of *Blastocystis* in asymptomatic individuals leading to the hypothesis that the organism is part of the healthy adult gut microbiota and that only specific STs and/or genotypes might be pathogenic (Chabe *et al.,* 2017; Scanlan and Stensvold, 2013; Scanlan *et al.,* 2014; Stensvold and van der Giezen, 2018). In this context, surveys examining prevalence of *Blastocystis* should ideally be

accompanied by subtyping. Thus, while the cosmopolitan status of *Blastocystis* is well established, geographic distribution of specific subtypes and their prevalence are relatively recent endeavors.

Research efforts on the latter two are disproportionate among countries and/or geographic regions: a plethora of *Blastocystis* studies come from the Americas and Europe, whilst Asia remains disproportionately sampled, given that half of the earth's population resides in Asian countries (El Safadi *et al.,* 2016; Scanlan *et al.,* 2016; Villegas-Gomez *et al.,* 2016). Collectively, these studies have shown that ST3 is the most prevalent one in almost all regions that have been examined, with a notable exception (Oliveira-Arbex *et al.,* 2018). Prevalence of the other common STs – namely ST1, ST2, and ST4 – seems to be dependent on geographic region, while ST6-ST9 are not as commonly encountered (Beghini *et al.,* 2017; Stensvold and Clark, 2016).

Herein, we collected samples from 178 asymptomatic adult humans living in Chiang Rai Province, Thailand and examined the prevalence, genetic diversity and distribution of *Blastocystis*. The region combines several particularities, which have created a culture and lifestyle that differs from other regions of Thailand. We provide the first subtyping data from the area, thus expanding knowledge on *Blastocystis* ST distribution and prevalence in the country.

2. Materials and Methods

2.1. Ethics statement

The human ethics committee of Mae Fah Luang University approved collection of fecal samples from adult Thai volunteers (License approval number

REH-60104). Volunteers signed an informed consent form before participating in the study.

2.2. Human subjects

All volunteers were over 18 years old, Thai and lived in Chiang Rai Province at the time of sampling (Fig. 1). In total, 178 adult volunteers participated in the study. The volunteers had no history of gastrointestinal symptoms, no diarrhea episodes a month prior to sampling and had received no antibiotic treatment at least two months prior to sample collection.

2.3. Stool collection and DNA extraction

Fecal samples were collected from the volunteers using sealed, sterile containers and stored at -80°C until DNA extraction. Samples were collected from six districts of Chiang Rai Province: Mae Chan (n=20), Mae Lao (n=20), Mae Sai (n=25), Muang (n=69), Pa Daet (n=23) and Phaya Mengrai (n=21) (Fig. 1). Total DNA was isolated using the QIAamp DNA Stool Mini Kit (Qiagen, Thailand) according to manufacturer's specifications. The extracted DNA was stored at -20 °C for later use.

2.4. PCR amplification, amplicon purification and sequencing

Polymerase chain reaction (PCR) was used to amplify a 650 bp fragment of the SSUrRNA at the 5′ of the gene. This fragment is considered the barcoding region of *Blastocystis* spp. (Scicluna *et al.,* 2006).

Two PCR reactions were used to amplify the SSUrRNA gene of *Blastocystis*. The broadly specific primers, RD5 5′ –

GGAAGCTTATCTGGTTGATCCTGCCAGTA – 3′ and RD3 5′ – GGGATCCTGATCCTTCCGCAGGTTCACCTAC – 3′ were used for the primary PCR reaction (Clark, 1997). The total volume of each reaction was 25µl containing the following reagents: 10X reaction buffer, 1 mM $MgCl_2$, 0.1 mM dNTPs, 0.2 µM forward primer, 0.2 µM reverse primer, 5U Taq DNA polymerase (RBC Bioscience, New Taipei City, Taiwan) and 12 µl HPLC grade water. Cycling conditions were as follows: initial denaturation at 94 °C for 3 min followed by 35 cycles of denaturation at 94 °C for 1 min 40 s, annealing at 65 °C for 1 min 40 s, extension at 72 °C for 1 min 40 s followed by a final extension at 72 °C for 10 min.

The more narrowly specific primers RD5F 5′ – ATCTGGTCGATCCTG CCAGT – 3′ and BhRDr 5′ – GAGCTTTTTAACTGCAACAACG – 3′ were employed for the secondary, nested PCR reaction. Reaction volume and concentration of reagents were as in the primary PCR with the exception of the DNA template. Instead of genomic fecal DNA, one µl from the primary PCR reaction was used. Cycling conditions were as follows: denaturation at 94 °C for 3 min, followed by 35 cycles of denaturation at 94 °C for 1 min, annealing at 55 °C for 1 min and extension at 72 °C for 1 min and a final extension at 72 °C for 10 min.

Positive PCR reactions were purified using the *HiYield™ Gel/PCR Fragments Extraction Kit* (RBC bioscience, USA) according to manufacturer's specifications. Purified PCR products were sequenced by 1stBASE, Malaysia.

2.5. Subtyping and phylogenetic analysis

The obtained sequences were examined and poor quality bases at the 5′ and 3′ends of the sequence were removed. Sequences were then subjected to BLAST search against GenBank database to exclude contamination. Sequences identified as

147 *Blastocystis*, were further checked against the Blastocystis Subtype Sequence Typing
148 (MLST) publicly available at http://pubmlst.org/blastocystis/, in order to identify
149 subtype and the corresponding allele.
150 The new sequences identified as *Blastocystis* along with sequences spanning
151 the breadth of diversity of *Blastocystis* (all subtypes and sequences from ectotherms)
152 were used to construct the dataset. In total, 103 sequences were aligned using MAFFT
153 v. 7 (Katoh and Toh, 2010), visually inspected and further improved manually.
154 Ambiguous regions were removed with trimAl v. 1.3 (Capella-Gutierrez *et al.*, 2009).
155 After trimming, the alignment contained 1,379 sites. A maximum likelihood (ML)
156 phylogenetic tree was constructed using RAxML v. 8 (Stamatakis, 2006) and the
157 general time reversible + Γ model of nucleotide substitution. The heuristic tree search
158 was based on 20 starting trees. One thousand bootstrap replicates were generated and
159 used for ML analysis. MrBayes v. 3.2.6 (Ronquist and Huelsenbeck, 2003) was used
160 to construct the Bayesian Inference (BI) tree using the same model as above. Two sets
161 of four independent Markov Chain Monte Carlo simulations were run for 1,500,000
162 generations. Sampling was done every 1,000 generations and 25% were discarded as
163 burn-in. Convergence was declared when the standard deviation of split sequences
164 was less than 0.01. Phylogenetic analyses were conducted on the Cipres Science
165 Gateway (http://www.phylo.org/portal2/home.action).
166 To further examine distribution of subtypes across Thailand, we downloaded
167 all *Blastocystis* sequences with the following identifiers: country =Thailand and
168 host=*Homo sapiens*. In total, 161 *Blastocystis* sequences were used for the analysis of
169 ST prevalence. For the ST distribution across Thailand 159 were used. Two sequences
170 were removed because the exact locality of collection was not noted.
171

172 # 3. Results

173

174 ## 3.1. Screening of fecal samples

175 Of the 178 samples, 41 (23%) were sequence positive for *Blastocystis* (Table
176 1). Differences were noted in the percent prevalence among districts. The highest
177 prevalence was observed in Phaya Mengrai District (33%), while the lowest was
178 noted in Mae Chan and Mae Lao Districts (15%).

179

180 ## 3.2. Subtype prevalence and distribution

181 *Blastocystis* prevalence in our study population was 23% (41/178). In total, six
182 of the nine STs that colonise humans were identified in this study: ST1 (n=7, 17%),
183 ST2 (n=1, 2%), ST3 (n=28, 68%), ST4 (n=1, 2%), ST6 (n=1, 2%) and ST7 (n=3, 7%).
184 Two alleles of ST1 were detected with the dominant one being allele 4, while four
185 alleles were found within the dominant ST3 (Fig. 2). Even though only three
186 sequences are available for ST7, two alleles were detected, suggesting a high degree
187 of genetic diversity for this ST in the study population. The prevalence of the various
188 *Blastocystis* subtypes in Thailand resembles the rest of the world (Fig. 3). Subtype 3 is
189 the most prevalent one, followed by ST1, ST2, ST6 and ST7. Nonetheless, notable
190 differences emerge when looking at the prevalence of STs according to geographic
191 region (Fig. 4).

192 All newly generated sequences have been submitted to GenBank (submission
193 number SUB3865516)

194 ## 3.3. Phylogenetic analyses

195 All newly derived sequences grouped within clades formed by previously
196 reported STs (Fig. 5). In agreement with previous studies, isolates from ectothermic

metazoans and those from ST15, ST16 and ST17 placed at a basal position (Betts *et al.*, 2018; Yoshikawa *et al.*, 2016). Placement of the new sequences confirmed ST predictions from the pubmlst website. The rest of the STs grouped into two large clusters, once consisting of ST3, ST4, ST8 and ST10 and the other formed by the remaining STs.

4. Discussion

All samples herein were collected from asymptomatic adults with no history of gastrointestinal disease. Our results match previous recent reports, which have demonstrated asymptomatic carriage of *Blastocystis* in populations from westernised and non-westernised countries, as well as, rural and urban settings (Lukeš *et al.*, 2015; Parfrey *et al.*, 2014; Scanlan *et al.*, 2014). Findings from analyses of large datasets are also consistent with these observations (Audebert *et al.*, 2016; Beghini *et al.*, 2017). These are in contrast with many previous studies that postulate links of *Blastocystis* with intestinal diseases and dysbiosis (Poirer *et al.*, 2012; Yakoob *et al.*, 2010). Thus, it is still unclear, whether *Blastocystis* is a pathogen, a harmless commensal or a beneficial member of gut microbiota. The debate has given rise to the speculation that pathogenicity of *Blastocystis* is limited to specific genotypes with the majority of them being harmless (Scanlan and Stensvold, 2013; Tan *et al.*, 2010). Given the polymicrobial nature of the gut, an as yet unexplored theme in the context of ecological theory, is whether *Blastocystis* becomes pathogenic through synergistic action with other microbes and/or their metabolic products. Nonetheless, the asymptomatic carriage of *Blastocystis* found in our study and others reinforces the idea of it being a part of the healthy gut microbiota.

We used PCR to amplify the barcoding region of *Blastocystis* (Scicluna *et al.*, 2006) from 178 fecal samples. This method demonstrated that the overall prevalence of *Blastocystis* in our study population was 23%. It is difficult to determine how this prevalence rate compares to other studies from Thailand. *Blastocystis* detection in the country is primarily based on microscopy (Boonjaraspinyo *et al.*, 2013; Kitvatanachai and Rhongbutsri, 2013; Kitvatanachai *et al.*, 2008; Ngrenngarmlert *et al.*, 2007; Pipatsatitpong *et al.*, 2012; Sagnuankiat *et al.*, 2014). In general, microscopy is less sensitive and underestimates prevalence. Molecular-based studies on *Blastocystis* constitute a recent development and remain extremely limited (Boondit *et al.*, 2014; Jantermtor *et al.*, 2013; Palasuwan *et al.*, 2016; Pintong *et al.*, 2014; Pipatsatitpong *et al.*, 2015; Popruk *et al.*, 2015; Thathaisong *et al.*, 2013). These molecular surveys have shown prevalence ranging from 6% to 51%. The observed variable data from our study and others could be the result of demographically different populations being surveyed. For instance, the highest abundance of *Blastocystis* was found in orphan male children, while residents of communities along Chao Praya River in Ayutthaya Province (central Thailand) had the lowest prevalence (Palasuwan *et al.*, 2016; Pintong *et al.*, 2014). Age, health status of the individuals, and general lifestyle are likely contributing factors accounting for the observed differences among studies (Leelayoova *et al.*, 2008).

Subtyping of *Blastocystis* positive samples identified six of the nine STs known to colonise humans: ST1, ST2, ST3, ST4, ST6 and ST7. ST3 was dominant and present in two thirds of the samples, followed by ST1 and ST7. This is slightly different than the global trend, whereby ST3, ST1 and ST2, in this order, are the most frequently encountered (Stensvold and Clark, 2016). This pattern is also noted in molecular surveys of *Blastocystis* in neighboring Southeast Asian countries (Laos,

Cambodia and Malaysia), except for Laos, where ST1 was the most frequent followed by ST3 and then ST2 (Nithyamathi *et al.,* 2016; Noradilah *et al.,* 2017; Sanpool *et al.,* 2017; Wang *et al.,* 2014). Herein, ST2, ST4 and ST6 were found in a single individual each. ST4 is a geography-influenced ST and is typically found in Europe, where it is abundant and highly prevalent (Alfellani *et al.,* 2013b; Beghini *et al.,* 2017; Forsell *et al.,* 2016;). Indeed, ST4 occurrence in Southeast Asia is extremely rare having been found so far only in Thailand and Malaysia (Nithyamathi *et al.,* 2016; Noradilah *et al.,* 2017; Popruk *et al.,* 2015).

When all available sequences from human stool samples from Thailand are taken into account (including ours), the most prevalent STs are ST3, ST1 and ST2, a pattern that follows the global trend (Stensvold and Clark, 2016). Given the known influence of geography on ST distribution, we zoomed into specific regions. Based on geography, Thailand is divided into four regions: north, northeast, center and south. Thus, when geographic region is taken into account, there are some notable differences in the ST distribution. For instance, the relative abundance of ST3 and ST1 changes. ST1 is dominant in the center, while ST3 dominates in the north and northeast. ST2 is absent in the northeast, whilst ST4 has only been found so far in the north. Importantly, the center, which is the most urbanised region of Thailand, has the least number of subtypes. Incidentally, this is also the most broadly surveyed region and where most sequences come from. Thus, though we cannot exclude the possibility that some STs circulate in the center and have yet to be detected, this is not very likely. Presence of ST6 and ST7 in the north and northeast could be attributed to domestic fowl, especially chickens and geese, which live in close proximity to humans.

The data obtained herein are the first on prevalence of *Blastocystis* and distribution of STs in asymptomatic individuals living in Chiang Rai Province. Our data along with other molecular data from Thailand will contribute to the knowledge on the epidemiology of *Blastocystis* in the country. Nonetheless, major gaps exist, in that: 1) molecular data on *Blastocystis* from Thailand is scant, thus ST distribution is largely unknown and, 2) the majority of Thai communities have yet to be sampled. For example, there is no molecular data of *Blastocystis* from the south part of Thailand. Moving forward, future studies should expand to generate subtyping information from more communities across Thailand and also include symptomatic individuals.

CONFLICTS OF INTEREST

The authors declare no conflict of interest

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FIGURE LEGENDS

Fig. 1. Map of Thailand and Chiang Rai Province districts. Sampling localities used in this investigation are depicted in red lettering.

Fig. 2. Frequency of *Blastocystis* alleles detected in the study population

Fig. 3. Prevalence of *Blastocystis* subtypes in Thailand (n=161)

Fig. 4. Prevalence and distribution of *Blastocystis* subtypes across geographic regions in Thailand

Fig. 5. Maximum likelihood phylogenetic tree inferred from 103 SSUrRNA sequences and 1379 sites. New sequences are depicted in bold lettering. Numerical values on the tree branches indicate bootstrap support percentages and posterior probabilities in this order.

Table(s)

Table 1. Prevalence of *Blastocystis* in six districts in Chiang Rai Province, Thailand

District	**No of samples**	**% prevalence (positives)**
Mae Chan	20	15 (3/20)
Mae Lao	20	15 (3/20)
Mae Sai	25	24 (6/25)
Muang	69	25 (17/69)
Pa Daet	23	22 (5/23)
Phaya Mengrai	21	33 (7/21)
Total	178	23 (41/178)

Figure(s)
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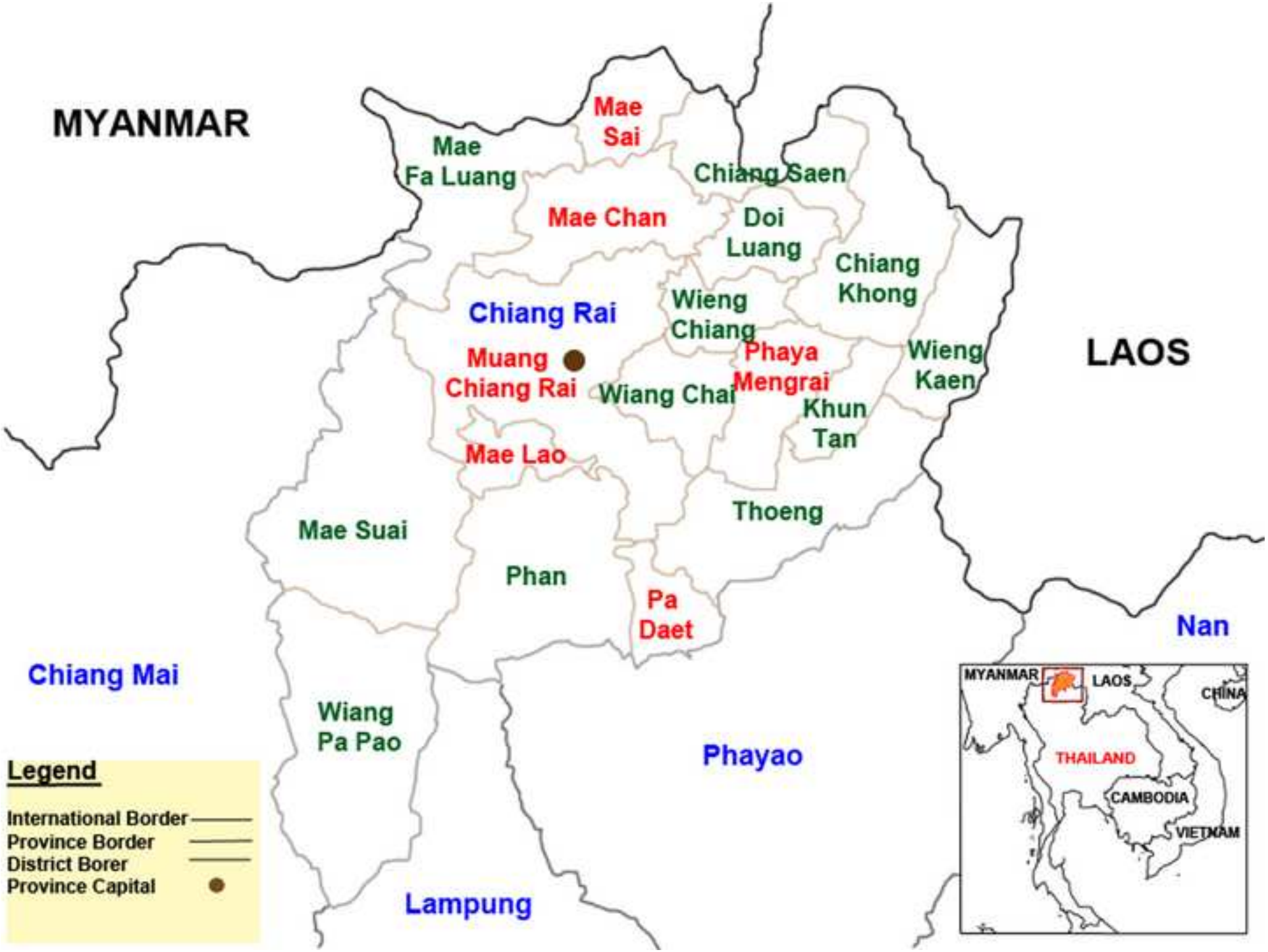

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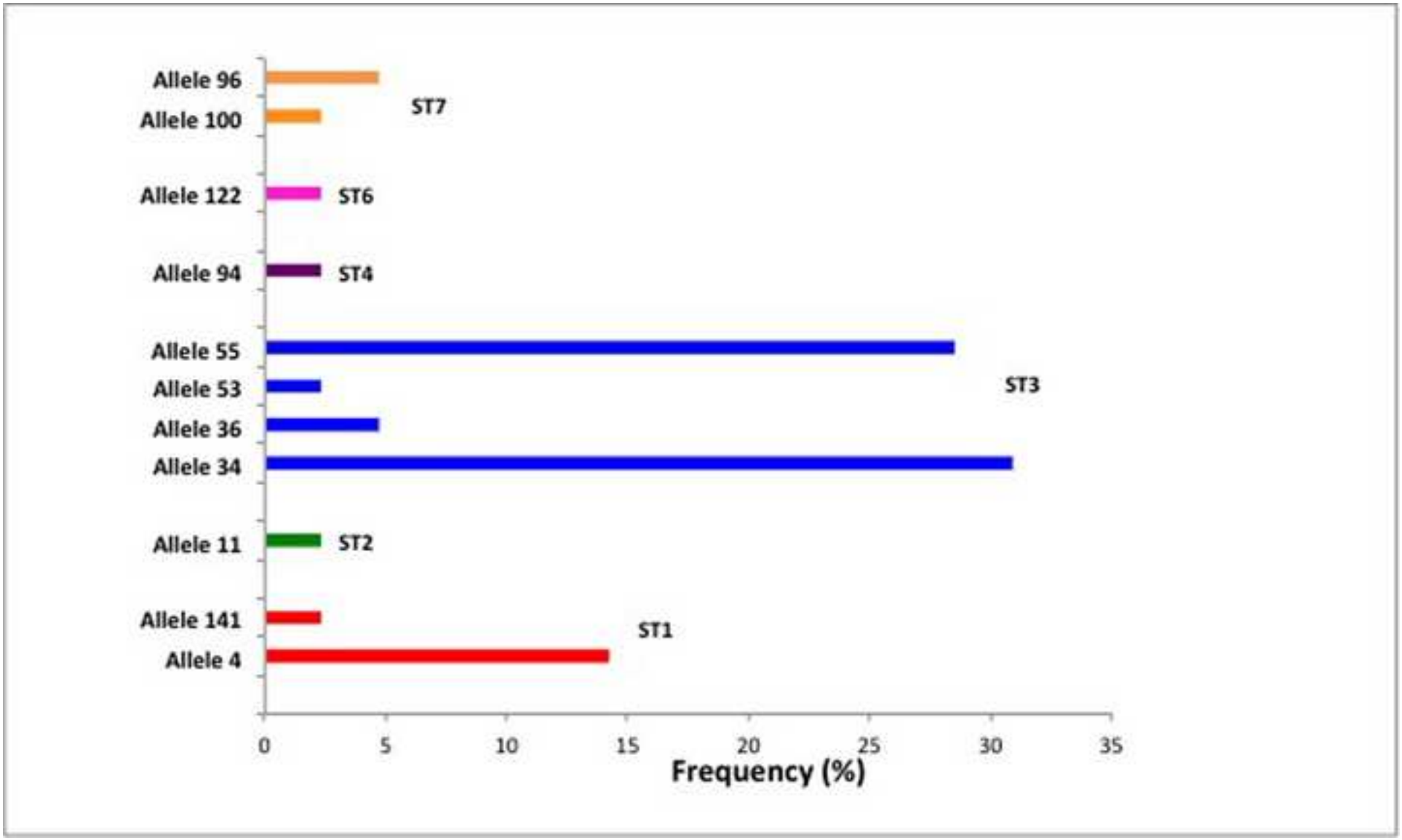

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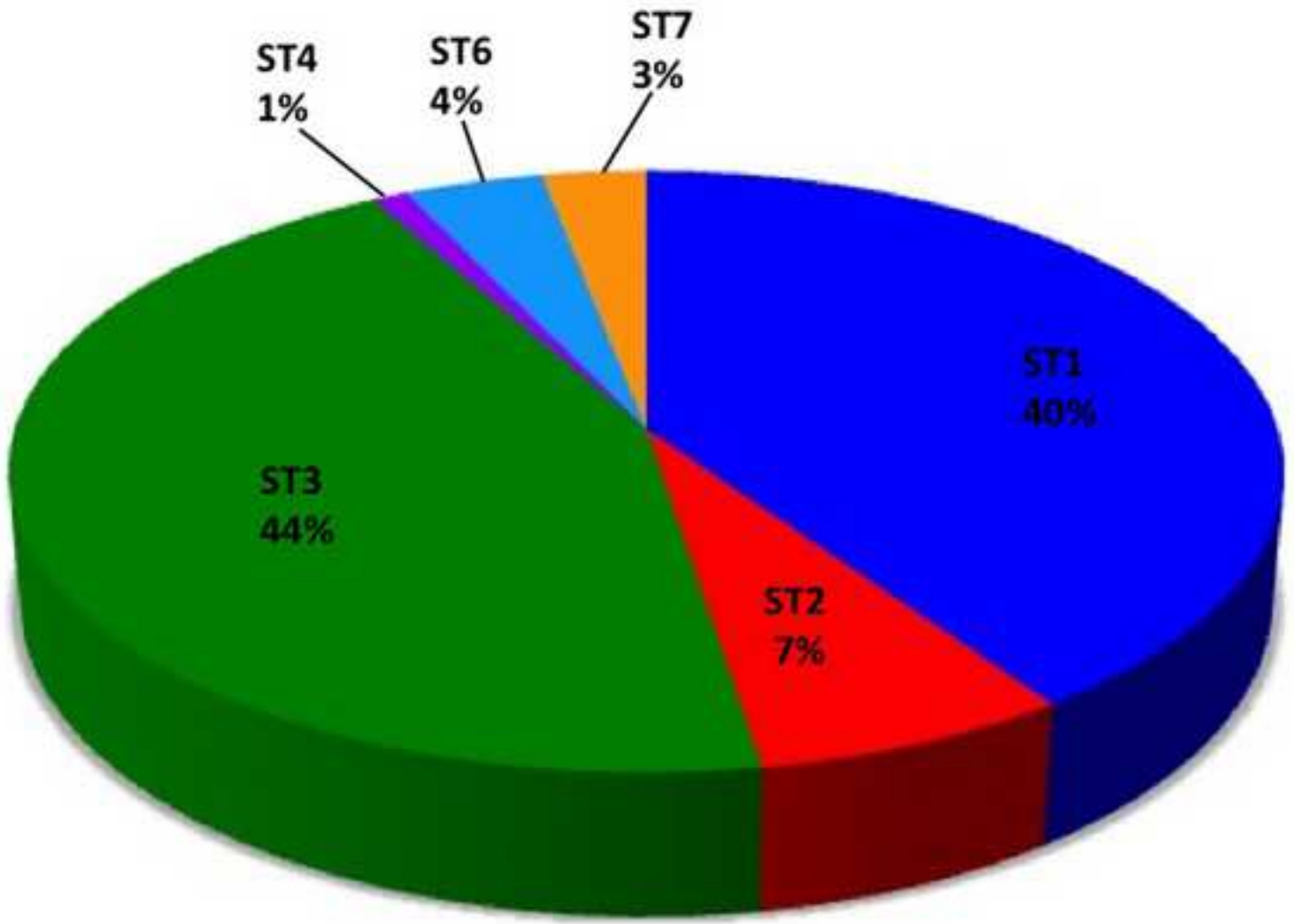

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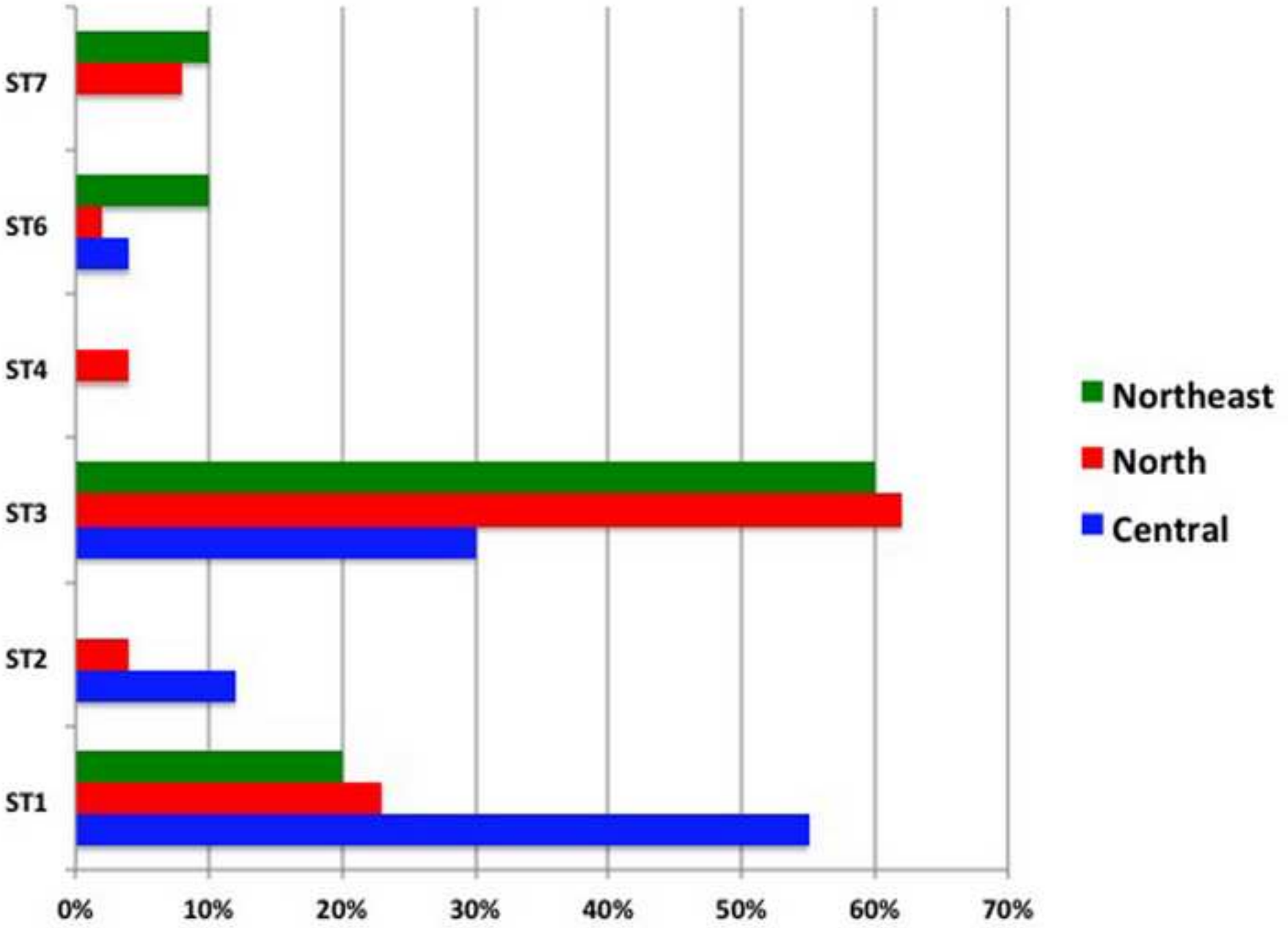

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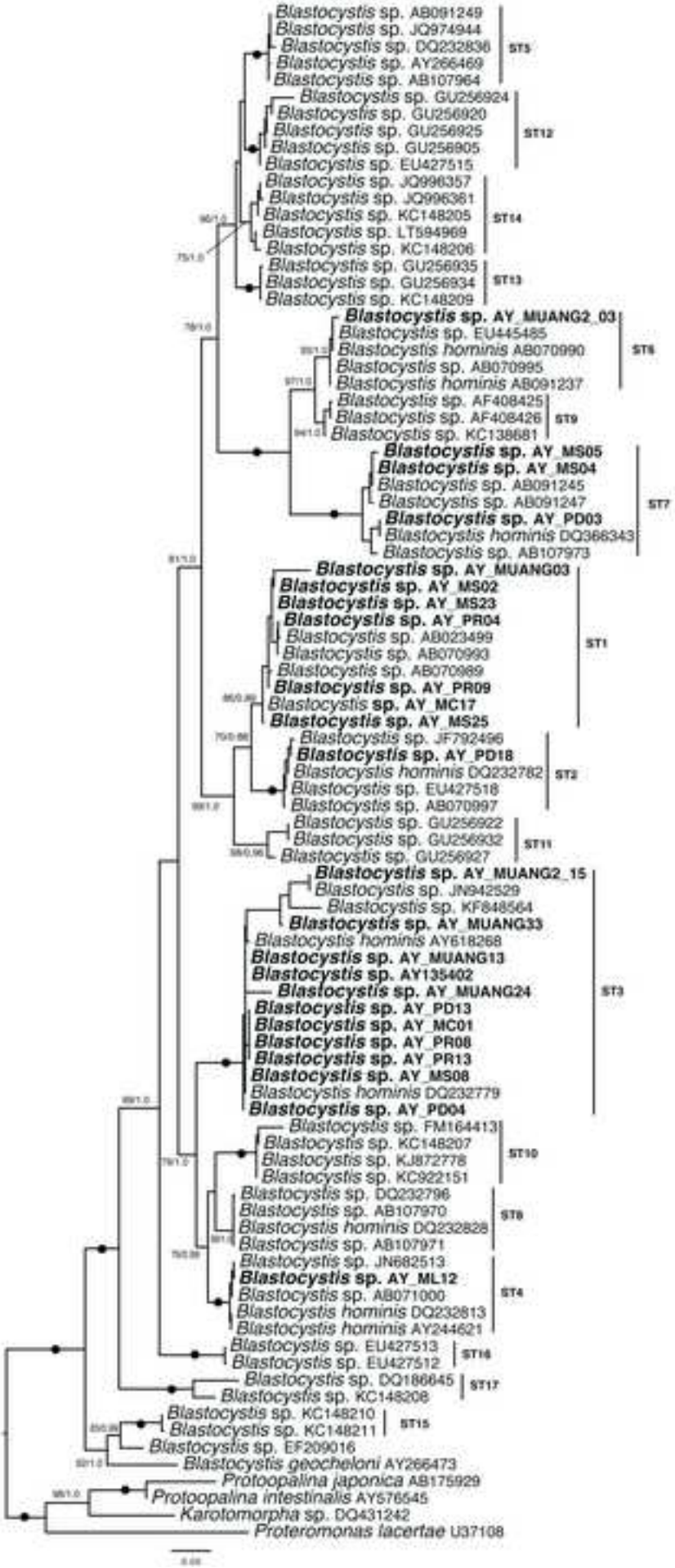

High diversity ~~and variable geographic distribution~~ of *Blastocystis* subtypes isolated from asymptomatic adults living in Chiang Rai, Thailand

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Running title: *Blastocystis* diversity and subtyping in Thai adults

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Abstract

Blastocystis is a common and broadly distributed microbial eukaryote inhabiting the gut of humans and other animals. The genetic diversity of *Blastocystis* is extremely high comprising no less than 17 subtypes in mammals and birds. Nonetheless, little is known about the prevalence and distribution of *Blastocystis* subtypes colonising humans in Thailand. Molecular surveys of *Blastocystis* remain extremely limited and usually focus on the central, urban part of the country. To address this knowledge gap, we collected stool samples from a population of Thai adults (n=178) residing in Chiang Rai Province. The barcoding region of the small subunit ribosomal RNA was employed to screen for *Blastocystis* and identify the subtype. Forty-one stool samples (23%) were identified as *Blastocystis* positive. Six of the nine subtypes that colonise humans were detected with subtype (ST) three being the most common (68%), followed by ST1 (17%) and ST7 (7%). Comparison of subtype prevalence across Thailand using all publicly available sequences showed that subtype distribution differs among geographic regions in the country. ST1 was most commonly encountered in the central region of Thailand, while ST3 dominated in the more rural north and northeast regions. ST2 was absent in the northeast, while ST7 was not found in the center. Thus, this study shows that ST prevalence and distribution differs not only among countries, but also among geographic regions within a country. Potential explanations for these observations are discussed herewith.

Keywords: *Blastocystis*; genetic diversity; prevalence; subtyping; Thailand

1. Introduction

Blastocystis is the most commonly found protist in the gastrointestinal tract of humans and other animals (Roberts *et al.*, 2013; Tan, 2008). Though *Blastocystis* exhibits morphological stasis, it has a high degree of genetic heterogeneity. Isolates from avian and mammalian hosts are~~The organism is~~ currently classified into 17 subtypes (STs) based on the divergence of the small subunit ribosomal RNA (SSU rRNA), though new STs might be arising (Alfellani *et al.*, 2013c; Betts *et al.*, 2018;). *Blastocystis* isolated from reptilian, amphibian and insect hosts are distinct and are not assigned into subtypes. So far, only ST1-ST9 have been found in humans with ST1-ST4 being the most common colonisers (Alfellani *et al.*, 2013b). However, these STs have also been detected in a variety of non-human hosts, suggesting that *Blastocystis* has low host specificity (Stensvold and Clark 2016). The exception is ST9, which has yet to be found in non-human hosts. The rest of the STs have so far been identified in both domesticated and wildlife animal hosts (Alfellani *et al.*, 2013a).

Lately, considerable research effort has gone into deciphering potential roles of *Blastocystis* in intestinal disease. For instance, an association between presence of *Blastocystis* and irritable bowel syndrome has been discussed frequently, though a definitive causal link has yet to be postulated (Jimenez-Gonzalez *et al.*, 2012; Khademvatan *et al.*, 2017; Poirer *et al.*, 2012; Yakoob *et al.*, 2010). Nonetheless, emerging data have shown presence of *Blastocystis* in asymptomatic individuals leading to the hypothesis that the organism is part of the healthy adult gut microbiota and that only specific STs and/or genotypes might be pathogenic (Chabe *et al.*, 2017; Scanlan and Stensvold, 2013; Scanlan *et al.*, 2014; Stensvold and van der Giezen, 2018). In this context, surveys examining prevalence of *Blastocystis* should ideally be

accompanied by subtyping. Thus, while the cosmopolitan status of *Blastocystis* is well established, geographic distribution of specific subtypes and their prevalence are relatively recent endeavors.

Research efforts on the latter two are disproportionate among countries and/or geographic regions: a plethora of *Blastocystis* studies come from the Americas and Europe, whilst Asia remains disproportionately sampled, given that half of the earth's population resides in Asian countries (El Safadi *et al.,* 2016; Scanlan *et al.,* 2016; Villegas-Gomez *et al.,* 2016). Collectively, these studies have shown that ST3 is the most prevalent one in almost all regions that have been examined, with a notable exception (Oliveira-Arbex *et al.,* 2018). Prevalence of the other common STs – namely ST1, ST2, and ST4 – seems to be dependent on geographic region, while ST6-ST9 are not as commonly encountered (Beghini *et al.,* 2017; Stensvold and Clark, 2016).

Herein, we collected samples from 178 asymptomatic adult humans living in Chiang Rai Province, Thailand and examined the prevalence, genetic diversity and distribution of *Blastocystis*. The region combines several particularities, which have created a culture and lifestyle that differs from other regions of Thailand. We provide the first subtyping data from the area, thus expanding knowledge on *Blastocystis* ST distribution and prevalence in the country.

2. Materials and Methods

2.1. Ethics statement

The human ethics committee of Mae Fah Luang University approved collection of fecal samples from adult Thai volunteers (License approval number

REH-60104). Volunteers signed an informed consent form before participating in the study.

2.2. Human subjects

All volunteers were over 18 years old, Thai and lived in Chiang Rai Province at the time of sampling (Fig. 1). In total, 178 adult volunteers participated in the study. The volunteers had no history of gastrointestinal symptoms, no diarrhea episodes a month prior to sampling and had received no antibiotic treatment at least two months prior to sample collection.

2.3. Stool collection and DNA extraction

Fecal samples were collected from the volunteers using sealed, sterile containers and stored at -80°C until DNA extraction. Samples were collected from six districts of Chiang Rai Province: Mae Chan (n=20), Mae Lao (n=20), Mae Sai (n=25), Muang (n=69), Pa Daet (n=23) and Phaya Mengrai (n=21) (Fig. 1). Total DNA was isolated using the QIAamp DNA Stool Mini Kit (Qiagen, Thailand) according to manufacturer's specifications. The extracted DNA was stored at -20 °C for later use.

2.4. PCR amplification, amplicon purification and sequencing

Polymerase chain reaction (PCR) was used to amplify a 650 bp fragment of the SSUrRNA at the 5′ of the gene. This fragment is considered the barcoding region of *Blastocystis* spp. (Scicluna *et al.,* 2006).

Two PCR reactions were used to amplify the SSUrRNA gene of *Blastocystis*. The broadly specific primers, RD5 5′ –

GGAAGCTTATCTGGTTGATCCTGCCAGTA – 3′ and RD3 5′ – GGGATCCTGATCCTTCCGCAGGTTCACCTAC – 3′ were used for the primary PCR reaction (Clark, 1997). The total volume of each reaction was 25µl containing the following reagents: 10X reaction buffer, 1 mM $MgCl_2$, 0.1 mM dNTPs, 0.2 µM forward primer, 0.2 µM reverse primer, 5U Taq DNA polymerase (RBC Bioscience, New Taipei City, Taiwan) and 12 µl HPLC grade water. Cycling conditions were as follows: initial denaturation at 94 °C for 3 min followed by 35 cycles of denaturation at 94 °C for 1 min 40 s, annealing at 65 °C for 1 min 40 s, extension at 72 °C for 1 min 40 s followed by a final extension at 72 °C for 10 min.

The more narrowly specific primers RD5F 5′ – ATCTGGTCGATCCTG CCAGT – 3′ and BhRDr 5′ – GAGCTTTTTAACTGCAACAACG – 3′ were employed for the secondary, nested PCR reaction. Reaction volume and concentration of reagents were as in the primary PCR with the exception of the DNA template. Instead of genomic fecal DNA, one µl from the primary PCR reaction was used. Cycling conditions were as follows: denaturation at 94 °C for 3 min, followed by 35 cycles of denaturation at 94 °C for 1 min, annealing at 55 °C for 1 min and extension at 72 °C for 1 min and a final extension at 72 °C for 10 min.

Positive PCR reactions were purified using the *HiYield™ Gel/PCR Fragments Extraction Kit* (RBC bioscience, USA) according to manufacturer's specifications. Purified PCR products were sequenced by 1stBASE, Malaysia.

2.5. Subtyping and phylogenetic analysis

The obtained sequences were examined and poor quality bases at the 5′ and 3′ends of the sequence were removed. Sequences were then subjected to BLAST search against GenBank database to exclude contamination. Sequences identified as

147 *Blastocystis*, were further checked against the Blastocystis Subtype Sequence Typing
148 (MLST) publicly available at http://pubmlst.org/blastocystis/, in order to identify
149 subtype and the corresponding allele.
150 The new sequences identified as *Blastocystis* along with sequences spanning
151 the breadth of diversity of *Blastocystis* (all subtypes and sequences from ectotherms)
152 were used to construct the dataset. In total, 103 sequences were aligned using MAFFT
153 v. 7 (Katoh and Toh, 2010), visually inspected and further improved manually.
154 Ambiguous regions were removed with trimAl v. 1.3 (Capella-Gutierrez *et al.*, 2009).
155 After trimming, the alignment contained 1,379 sites. A maximum likelihood (ML)
156 phylogenetic tree was constructed using RAxML v. 8 (Stamatakis, 2006) and the
157 general time reversible + Γ model of nucleotide substitution. The heuristic tree search
158 was based on 20 starting trees. One thousand bootstrap replicates were generated and
159 used for ML analysis. MrBayes v. 3.2.6 (Ronquist and Huelsenbeck, 2003) was used
160 to construct the Bayesian Inference (BI) tree using the same model as above. Two sets
161 of four independent Markov Chain Monte Carlo simulations were run for 1,500,000
162 generations. Sampling was done every 1,000 generations and 25% were discarded as
163 burn-in. Convergence was declared when the standard deviation of split sequences
164 was less than 0.01. Phylogenetic analyses were conducted on the Cipres Science
165 Gateway (http://www.phylo.org/portal2/home.action).
166 To further examine distribution of subtypes across Thailand, we downloaded
167 all *Blastocystis* sequences with the following identifiers: country =Thailand and
168 host=*Homo sapiens*. In total, 161 *Blastocystis* sequences were used for the analysis of
169 ST prevalence. For the ST distribution across Thailand 159 were used. Two sequences
170 were removed because the exact locality of collection was not noted.
171

172 # 3. Results

173

174 ## 3.1. Screening of fecal samples

175 Of the 178 samples, 41 (23%) were sequence positive for *Blastocystis* (Table
176 1). Differences were noted in the percent prevalence among districts. The highest
177 prevalence was observed in Phaya Mengrai District (33%), while the lowest was
178 noted in Mae Chan and Mae Lao Districts (15%).

179

180 ## 3.2. Subtype prevalence and distribution

181 *Blastocystis* prevalence in our study population was 23% (41/178). In total, six
182 of the nine STs that colonise humans were identified in this study: ST1 (n=7, 17%),
183 ST2 (n=1, 2%), ST3 (n=28, 68%), ST4 (n=1, 2%), ST6 (n=1, 2%) and ST7 (n=3, 7%).
184 Two alleles of ST1 were detected with the dominant one being allele 4, while four
185 alleles were found within the dominant ST3 (Fig. 2). Even though only three
186 sequences are available for ST7, two alleles were detected, suggesting a high degree
187 of genetic diversity for this ST in the study population. The prevalence of the various
188 *Blastocystis* subtypes in Thailand resembles the rest of the world (Fig. 3). Subtype 3 is
189 the most prevalent one, followed by ST1, ST2, ST6 and ST7. Nonetheless, notable
190 differences emerge when looking at the prevalence of STs according to geographic
191 region (Fig. 4).

192 All newly generated sequences have been submitted to GenBank (submission
193 number SUB3865516)

194 ## 3.3. Phylogenetic analyses

195 All newly derived sequences grouped within clades formed by previously
196 reported STs (Fig. 5). In agreement with previous studies, isolates from ectothermic

metazoans and those from ST15, ST16 and ST17 placed at a basal position (Betts *et al.*, 2018; Yoshikawa *et al.*, 2016). Placement of the new sequences confirmed ST predictions from the pubmlst website. The rest of the STs grouped into two large clusters, once consisting of ST3, ST4, ST8 and ST10 and the other formed by the remaining STs.

4. Discussion

All samples herein were collected from asymptomatic adults with no history of gastrointestinal disease. Our results match previous recent reports, which have demonstrated asymptomatic carriage of *Blastocystis* in populations from westernised and non-westernised countries, as well as, rural and urban settings (Lukeš *et al.*, 2015; Parfrey *et al.*, 2014; Scanlan *et al.*, 2014). Findings from analyses of large datasets are also consistent with these observations (Audebert *et al.*, 2016; Beghini *et al.*, 2017). These are in contrast with many previous studies that postulate links of *Blastocystis* with intestinal diseases and dysbiosis (Poirer *et al.*, 2012; Yakoob *et al.*, 2010). Thus, it is still unclear, whether *Blastocystis* is a pathogen, a harmless commensal or a beneficial member of gut microbiota. The debate has~~These contrary findings have~~ given rise to the speculation that pathogenicity of *Blastocystis* is limited to specific genotypes with the majority of them being harmless (Scanlan and Stensvold, 2013; Tan *et al.*, 2010). Given the polymicrobial nature of the gut, an as yet unexplored theme in the context of ecological theory, is whether *Blastocystis* becomes pathogenic through synergistic action with other microbes and/or their metabolic products. Nonetheless, the asymptomatic carriage of *Blastocystis* found in our study and others reinforces the idea of it being a part of the healthy gut microbiota.

We used PCR to amplify the barcoding region of *Blastocystis* (Scicluna *et al.*, 2006) from 178 fecal samples. This method demonstrated that the overall prevalence of *Blastocystis* in our study population was 23%. It is difficult to determine how this prevalence rate compares to other studies from Thailand. *Blastocystis* detection in the country is primarily based on microscopy (Boonjaraspinyo *et al.*, 2013; Kitvatanachai and Rhongbutsri, 2013; Kitvatanachai *et al.*, 2008; Ngrenngarmlert *et al.*, 2007; Pipatsatitpong *et al.*, 2012; Sagnuankiat *et al.*, 2014). In general, microscopy is less sensitive and underestimates prevalence. Molecular-based studies on *Blastocystis* constitute a recent development and remain extremely limited (Boondit *et al.*, 2014; Jantermtor *et al.*, 2013; Palasuwan *et al.*, 2016; Pintong *et al.*, 2014; Pipatsatitpong *et al.*, 2015; Popruk *et al.*, 2015; Thathaisong *et al.*, 2013). These molecular surveys have shown prevalence ranging from 6% to 51%. The observed variable data from our study and others could be the result of demographically different populations being surveyed. For instance, the highest abundance of *Blastocystis* was found in orphan male children, while residents of communities along Chao Praya River in Ayutthaya Province (central Thailand) had the lowest prevalence (Palasuwan *et al.*, 2016; Pintong *et al.*, 2014). Age, health status of the individuals, and general lifestyle are likely contributing factors accounting for the observed differences among studies (Leelayoova *et al.*, 2008).

Subtyping of *Blastocystis* positive samples identified six of the nine STs known to colonise humans: ST1, ST2, ST3, ST4, ST6 and ST7. ST3 was dominant and present in two thirds of the samples, followed by ST1 and ST7. This is slightly different than the global trend, whereby ST3, ST1 and ST2, in this order, are the most frequently encountered (Stensvold and Clark, 2016). This pattern is also noted in molecular surveys of *Blastocystis* in neighboring Southeast Asian countries (Laos,

Cambodia and Malaysia), except for Laos, where ST1 was the most frequent followed by ST3 and then ST2 (Nithyamathi *et al.*, 2016; Noradilah *et al.*, 2017; Sanpool *et al.*, 2017; Wang *et al.*, 2014). Herein, ST2, ST4 and ST6 were found in a single individual each. ST4 is a geography-influenced ST and is typically found in Europe, where it is abundant and highly prevalent (Alfellani *et al.*, 2013b; Beghini *et al.*, 2017; Forsell *et al.*, 2016;). Indeed, ST4 occurrence in Southeast Asia is extremely rare having been found so far only~~. In molecular surveys taking place~~ in Thailand ~~only two cases of ST4 have been found: one in this study and another from Tak, a northwestern province bordering Myanmar (Popruk *et al.*, 2015). In the neighboring countries of Laos, Cambodia,~~ and Malaysia~~, ST4 has been found only in Malaysia~~ (Nithyamathi *et al.*, 2016; Noradilah *et al.*, 2017; Popruk *et al.*, 2015).~~). Regrettably, there is no publicly available data on *Blastocystis* from Myanmar. Alfellani *et al.*, (2013b), noted that ST4 occurs rarely in places, where ST1 dominates, and/or in regions where Muslim populations predominate, an observation that has since been reinforced (Forsell *et al.*, 2016). Another explanation could be that the Thai ST4 cases represent imports from Europe. Though none of the participants in our study travelled to Europe, it is unknown, if other household members did. If this indeed were the case, it would be interesting to see how the newly arrived STs affect the resident microbiota.~~

When all available sequences from human stool samples from Thailand are taken into account (including ours), the most prevalent STs are ST3, ST1 and ST2, a pattern that follows the global trend (Stensvold and Clark, 2016). Given the known influence of geography on ST distribution, we zoomed into specific regions. Based on geography, Thailand is divided into four regions: north, northeast, center and south. Thus, when geographic region is taken into account, there are some notable differences in the ST distribution. For instance, the relative abundance of ST3 and

ST1 changes. ST1 is dominant in the center, while ST3 dominates in the north and northeast. ST2 is absent in the northeast, whilst ST4 has only been found so far in the north. Importantly, the center, which is the most urbanised region of Thailand, has the least number of subtypes. Incidentally, this is also the most broadly surveyed region and where most sequences come from. Thus, though we cannot exclude the possibility that some STs circulate in the center and have yet to be detected, this is not very likely. Presence of ST6 and ST7 in~~Explaining these differences is a challenging endeavor. It is known that the four geographic regions of Thailand also happen to be gastronomically distinct. In particular,~~ the north and northeast could be attributed to domestic fowl,~~, and most~~ especially chickens and geese, which live in close proximity to humans~~Chiang Rai is of interest, because of its northernmost location (Fig. 1) and the mixed influence of nearby Laos and Myanmar on daily life, including diet. In a broader context, and given the influence of diet on microbial communities, the observed differences in ST distribution could also reflect differences in gut microbiota composition. In that vein, recent data have demonstrated that *Blastocystis* is more prevalent in individuals with increased *Prevotella* and *Ruminococcus* and decreased *Bacteroides* (Andersen and Stensvold, 2015; Audebert *et al.*, 2016; Beghini *et al.*, 2017; O'Brien Andersen *et al.*, 2016;). Nonetheless, a knowledge gap exists, as to whether similar differences drive prevalence of specific STs and vice versa~~.

The data obtained herein are the first on prevalence of *Blastocystis* and distribution of STs in asymptomatic individuals living in Chiang Rai Province.~~, and one of the few in Thailand.~~ Our data along with other molecular data from Thailand will contribute to the knowledge on the epidemiology of *Blastocystis* in the country. Nonetheless, major gaps exist, in that: 1) molecular data on *Blastocystis* from Thailand is scant, thus ST distribution is largely unknown and, 2) the majority of Thai

297 communities have yet to be sampled. For example, there is no molecular data of
298 *Blastocystis* from the south part of Thailand. Moving forward, future studies should
299 expand to generate subtyping information from~~and include~~ more communities
300 across~~not only from~~ Thailand and also include symptomatic individuals~~, but~~
301 ~~neighboring countries as well~~.
302
303 **CONFLICTS OF INTEREST**
304 The authors declare no conflict of interest
305
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487

FIGURE LEGENDS

Fig. 1. Map of Thailand and Chiang Rai Province districts. Sampling localities used in this investigation are depicted in red lettering.

Fig. 2. Frequency of *Blastocystis* alleles detected in the study population

Fig. 3. Prevalence of *Blastocystis* subtypes in Thailand (n=161)

Fig. 4. Prevalence and distribution of *Blastocystis* subtypes across geographic regions in Thailand

Fig. 5. Maximum likelihood phylogenetic tree inferred from 103 SSUrRNA sequences and 1379 sites. New sequences are depicted in bold lettering. Numerical values on the tree branches indicate bootstrap support percentages and posterior probabilities in this order.