Heliyon 10 (2024) e29019

Contents lists available at ScienceDirect

Heliyon

journal homepage: www.cell.com/heliyon

Research article

Subtype–host patterns and genetic differentiation of *Blastocystis* sp. in the Philippines

Davin Edric V. Adao, Windell L. Rivera *

Pathogen–Host–Environment Interactions Research Laboratory, Institute of Biology, College of Science, University of the Philippines Diliman, Quezon City, 1101, Philippines

ARTICLE INFO

Keywords:
Allele
Blastocystis
Diversity
Host specificity
Subtype

ABSTRACT

Blastocystis sp. is a gastrointestinal protozoan commonly encountered in humans and animals. Specificity to certain hosts may be associated with 38 known subtypes (STs) and 8 non-mammalian and avian STs (NMASTs). This can be determined by analyzing ST–host associations, ST–allele data, genetic variability analyses, and fixation index (F_{ST}) with sufficient data present. Thus, newly acquired and previously published data on *Blastocystis* sp. STs and NMASTs from the Philippines were compiled to determine the following: (1) ST–host associations, (2) ST–allele diversity per ST in certain hosts/sources, (3) intrasubtype diversity of certain STs found in different hosts using genetic variability analysis, and (4) comparison of similarities between specific ST populations to determine if these are the same circulating populations using F_{ST}. A total of 448 samples subtyped using both sequence-tagged site primers and the 600-bp barcoding region of the *Blastocystis* sp. SSU rRNA gene were analyzed in this study. Patterns of association for the Philippine samples were similar to those from neighboring Southeast Asian countries and around the world: ST1–ST4 were found in humans but ST3 was the most common, ST5 were found in pigs, and ST6 and ST7 were found in poultry. *Blastocystis* sp. from humans are mostly the same ST alleles (ST3 allele 34 and ST1 allele 4) while 3–5 ST alleles were found in the most common STs in pigs, macaques, and poultry. Also, ST1, ST3, ST5, and NMAST I are undergoing population expansion according to genetic variability analyses through possible addition of new alleles based on ST–allele diversity. Moreover, F_{ST} shows the same circulating population of ST1 in humans, pigs, and water indicating a possible waterborne route of cross-transmission. In contrast, ST3 found in humans possibly come from the same circulating population and is genetically distinct from those in nonhuman sources.

1. Introduction

Blastocystis sp. is a gastrointestinal protozoan commonly encountered in humans and animals with unclear pathogenicity. Currently, there are 38 recognized subtypes (STs) based on SSU rRNA gene sequence variations designated as ST1–ST17 [1–4], ST21, ST23–ST29 [5], ST30, ST31 [6], ST32 [7], and ST35–ST38 [8]. ST21 and ST23–ST26 are considered new STs after further analysis by sequencing more than 80% of the SSU rRNA gene. There are also 8 nonmammalian and avian STs (NMASTs) identified based on the phylogenetic studies by Cian et al. [9] and Yoshikawa et al. [10] that cluster with the mammalian and avian STs [11]. These STs and

* Corresponding author.
E-mail address: wlrivera@science.upd.edu.ph (W.L. Rivera).

https://doi.org/10.1016/j.heliyon.2024.e29019
Received 20 September 2023; Received in revised form 27 March 2024; Accepted 28 March 2024
Available online 3 April 2024

NMASTs can also be identified using the 5′-end 600-bp barcoding region of the SSU rRNA gene [12] and even assigned alleles based on this barcoding region [13]. Genetic variability analyses of the segments of the SSU rRNA gene or ITS region of *Blastocystis*, such as nucleotide diversity and population fixation index (F_{ST}), have also found its utility in assessing differences between similar ST populations found in different host populations [14–17]. For example, *Blastocystis* sp. in children from two cities in Mexico – an arid city and a humid subtropical city – were found to be genetically distinct from each other, whereas those from the adults did not indicate cross-transmission of the organisms between adult populations [17]. The importance of the genetic and population analyses of *Blastocystis* sp. lies in their possible association with host specificity or to certain populations of the host. In general, *Blastocystis* sp. has low host specificity [18,19], but certain STs are most commonly encountered in particular hosts such as ST3 in humans, ST5 in pigs, and ST7 in birds [1,4]. ST1–ST4 are the most commonly encountered STs in human patients with or without symptoms of a disease such as irritable bowel syndrome [3]. This study aimed to analyze the *Blastocystis* sp. ST–host association in the Philippines as well as possible intrasubtype diversity or intrasubtype patterns of distribution.

In this study, data on presence of *Blastocystis* sp. STs and their respective hosts from the Philippines were compiled along with new sequences. These include both SSU rRNA gene sequence data and sequence-tagged site (STS) data. This is to determine which *Blastocystis* STs are found in different hosts (human or animal) and sources (water sources) from the Philippines. The ST–alleles were also identified for the SSU rRNA gene data to determine which alleles are present in *Blastocystis* samples from the Philippines. Genetic variability measures were then used to determine genetic diversity of certain STs and NMASTs and if these populations are more likely neutral or undergoing population expansion or reduction. Finally, F_{ST} was used to determine if certain populations of *Blastocystis* sp. ST1 and ST3 were differentiated or overlapped between common hosts or sources. This also indicated if these populations are genetically similar or distinct as an indicator of possible intrasubtype host specificity. Studies on *Blastocystis* sp. in the Philippines have mostly been on ST identification of samples from humans, animals, and water samples [19–24]. Compiling all the data currently available in the Philippines and adding more sequences can reveal the common patterns of *Blastocystis* ST–host distribution. Identification of intrasubtype diversity such as alleles and genetic variability measures as well as F_{ST} can further elaborate on whether the same circulating populations of *Blastocystis* sp. are found in different hosts or sources. These in turn can further discern patterns of cross-transmission or host specificity.

2. Materials and methods

2.1. Blastocystis sp. from stool samples

Stool samples were collected from chickens (n = 13), ducks (n = 19), turkeys (n = 23), and goats (n = 11) from a farm in Tanay, Rizal; macaques (n = 63) from the Quezon City Parks and Wildlife Bureau (PAWB); a pig from Laguna (n = 1); chickens from Quezon City (n = 5); and toads from Quezon City (n = 64). These samples were inoculated onto a biphasic medium consisting of 1.5% agar overlaid with liquid media and supplemented with 10% horse serum and penicillin–streptomycin [19] and incubated for 3–5 days at 37 °C. Positive samples were identified by microscopy and maintained by subculturing in the same medium every 3–5 days and incubation at 37 °C.

2.2. Blastocystis sp. from cockroach samples

Cockroaches (n = 127) were captured from residential areas in Quezon City. *Blastocystis* sp. was collected by inoculating the gut contents of cockroaches dissected after chloroform treatment similar to the method of Zaman et al. [25]. Gut contents were inoculated and sub-cultured into the same biphasic medium as described above and under the same conditions as those of the animal stool samples. Positive samples were identified by microscopy and sub-cultured every 3–5 days.

2.3. Blastocystis sp. from water samples

A total of 51 water samples were taken particularly from flood-prone areas and/or areas with dense populations of people that may come into contact with the water. These samples were taken from various sources in the National Capital Region (n = 35) and the provinces of Laguna (n = 8), Batangas (n = 2), Bulacan (n = 5), and Rizal (n = 1). Sources included creeks (n = 24), rivers (n = 16), lake (n = 2), industrial wastewater (n = 7), and floodwater (n = 2). Approximately 300 mL of water was collected in sterile glass bottles, and the water debris was collected within 24 h. Water samples were centrifuged at 3000 rpm for 10 min repeatedly in 50-mL tubes until the volume was reduced to 5 mL, containing the sediments and debris. Pellets were resuspended, and 100 μL of suspended sediments was used for inoculation. Similar media and subculture conditions as stated above were followed. Positive samples were identified by microscopy and sub-cultured every 3–5 days.

2.4. DNA extraction

DNA extraction of culture-positive samples was performed after at least two subcultures to reduce contaminants. *Blastocystis* sp. cells at the bottom of the biphasic medium were collected and washed thrice in phosphate-buffered saline (PBS) by centrifugation at 10,000 rpm for 2 min. Pellets were mixed with 200 μL of 5% Chelex-100 and 100 μL of sterile distilled water and incubated for 30 min at 56 °C following the protocol of Rivera and Ong [26]. Samples were then briefly vortexed and subjected to an 8-min boiling water bath before centrifugation at 13,000 rpm for 2 min to collect the DNA suspended in the supernate. DNA extracts were stored at 4 °C

Table 1
Summary of *Blastocystis* SSU rRNA sequences from the Philippines used in this study.

Host/source	Subtype (n)	Location	Description	Publication	GenBank accession number
Chicken	ST6 (1)	Batangas	Farm animal	[19]	EU445485
Pig	ST1 (1)	Batangas	Farm animal		EU445486
	ST2 (1)				EU445487
Macaque	ST1 (2)	Rizal	Captive zoo animal		EU445488, EU445490
	ST2 (1)				EU445491
	ST3 (1)				EU445489
Human	ST1 (1)	Rizal	Asymptomatic residents		EU445492
	ST3 (4)				EU445493–EU445496
Wastewater	ST1 (7)	Metro Manila, Aklan	Near shopping mall, city jail, shopping mall, hotel/resort, and residential areas	[22]	GU992411, GU992413–GU992417, GU992419
	ST2 (2)	Metro Manila, Rizal	Near residential area and zoo		GU992412, GU992418
Human	ST3 (2)	Metro	Asymptomatic zookeepers	This study	JF750333, KY610164
Terrapene carolina (box turtle)	NMAST I	Manila	Captive zoo animal	This study	JF750335
Pig	ST1 (10)	Laguna	Farm animal	[21]	KP233714–KP233722, KP233731
	ST5 (8)		Farm animal		KP233723–KP233729, KP233740
	ST7 (1)		Farm animal		KP233734
Goat	ST14 (1)		Farm animal		KP233738
Chicken	ST7 (4)		Farm animal		KP233730, KP233732–KP233733, KP233736
Duck	ST7 (2)		Farm animal		KP233735, KP233737
	NMAST I (1)		Farm animal		KP233739
Human	ST1 (9)	Metro Manila	Asymptomatic residents	[20]	KP408441, KP408445, KP408451–KP408452, KT374017–KT374021
	ST3 (19)		Asymptomatic residents		KP404444–KP408439, KP408442–KP408443, KP408446–KP408450, KP408453, KT374022–KT374026
	ST4 (1)		Asymptomatic residents		KP408440
Pig	ST1 (3)	Metro	Farm animal	[24]	KT374035, KT374037, KT374039
	ST5 (11)	Manila	Farm animal		KT374027–KT374034, KT374036, KT374038, KT374040
Human	ST1 (3)	Rizal	Asymptomatic residents	[29]	KY610125, KY610128, KY610131
	ST3 (11)		Asymptomatic residents		KY610126–KY610130, KY610132–KY610137
Human	ST1 (2)	Metro	Asymptomatic residents	This study	KY610144, KY610150
	ST3 (15)	Manila	Asymptomatic residents, Patients with gastrointestinal symptoms		KY610138–KY610143, KY610145–KY610149, KY610151–KY610154
Human	ST1 (1)	Rizal	Asymptomatic residents	This study	KY610159
	ST2 (2)		Asymptomatic residents		KY610155–KY610156
	ST3 (3)		Asymptomatic residents		KY610157–KY610158, KY610160
	ST3 (3)	Batangas	Asymptomatic residents		KY610161–KY610163
Human	ST1 (1)	Laguna	Asymptomatic residents	This study	KY610165
	ST3 (1)		Asymptomatic residents		KY610166
Pig	ST1 (2)	Bulacan	Farm animal	[31]	KY610167, KY610194
	ST2 (1)		Farm animal		KY610196
	ST3 (1)		Farm animal		KY610169
	ST5 (31)		Farm animal		KY610168, KY610170–KY610193, KY610195, KY610197, MF737387–MF737390
Pig	ST1 (1)	Batangas	Farm animal	This study	KY610205
	ST2 (2)		Farm animal		KY610204
	ST3 (3)		Farm animal		KY610203
	ST5 (5)		Farm animal		KY610198–KY610202
Macaque	ST3 (3)	Rizal	Captive zoo animal		KY929118–KY929120
Chicken	ST7 (7)	Batangas	Farm animal		KY964535–KY964539
Macaque	ST1 (15)	Metro	Captive zoo animal	This study	KY929102–KY929104, KY929106–KY929117
	ST3 (3)	Manila	Captive zoo animal		KY929101, KY929105, KY929104
	NMAST I (1)		Captive zoo animal		MF737391
Chicken	ST6 (1)	Rizal	Farm animal	This study	KY964531
	ST7 (3)		Farm animal		KY964532–KY964534
Duck	ST7 (13)		Farm animal		KY964518–KY964530
Turkey	ST6 (4)		Farm animal		KY964511, KY964513–KY964514, KY964516
	ST7 (4)		Farm animal		KY964510, KY964512, KY964515, KY964517

(*continued on next page*)

Table 1 (*continued*)

Host/source	Subtype (n)	Location	Description	Publication	GenBank accession number
Creek water	ST1 (3)	Metro Manila	Within school area	This study	KY964540, KY964541, MF737397
	ST3 (1)	Bulacan	Near hotel/resort		MF737396
River water	ST1 (2)	Metro Manila	Near residential area		KY964542, MF737392
	ST4 (1)		Near residential area		MF737395
	ST1 (2)	Cavite	Near residential area		MF737393–MF737394
Lake water	ST1 (2)	Laguna	Near residential and commercial areas		MH100671, MH100673
	ST2 (1)		Near residential and commercial areas		MH100669
	ST3 (1)		Near residential and commercial areas		MH100672
Floodwater	ST1 (1)	Metro Manila	Near residential area		MH100670
Pig	ST3 (1)	Laguna	Farm animal	This study	KY964545
	ST5 (1)		Farm animal		OR352498
	ST7 (1)		Farm animal		KY964546
Chicken	ST7 (1)	Metro Manila	Farm animal	This study	OR352501
Cockroach	NMAST VI (1)	Metro Manila	Captured animal	This study	KY964543
Dog	ST1 (1)	Metro Manila	Pet	This study	KY964544
Toad	NMAST I (10)	Metro Manila	Captured animal	This study	OR458318–OR458327

prior to the amplification of the barcode SSU rRNA gene region.

2.5. Polymerase chain reaction (PCR) and sequencing

PCR was performed using the primers RD5 and BhRDr following the protocol of Scicluna et al. [12] to amplify the 600-bp barcoding region of the *Blastocystis* sp. SSU rRNA gene. Stored DNA extracts previously verified by sequencing as *Blastocystis* and published were used as positive controls while PCR mix without added DNA extracts were used as negative controls. PCR products were sent to Macrogen (Seoul, Korea) or the Philippine Genome Center for purification and sequencing. Forward and reverse sequences were aligned using the ClustalW function of BioEdit v.7 [27] and by visual inspection. Sequences were verified as *Blastocystis* sp. by uploading onto the BLAST website (https://blast.ncbi.nlm.nih.gov/). STs and alleles were identified by uploading sequences onto the *Blastocystis* sp. PubMLST website (https://pubmlst.org/blastocystis/). Sequences were verified as *Blastocystis* sp. if results were 97%–100% identical to sequences stored in GenBank. Alleles were considered only if sequences were exact matches with stored sequences in the *Blastocystis* sp. PubMLST database. Otherwise, the closest match was indicated. Samples with sequences 89%–90% similar to *Blastocystis* sp. due to unclear chromatograms were considered mixed ST cultures. All new sequences were submitted to GenBank.

2.6. Stored DNA extracts

Stored DNA extracts of *Blastocystis* from previous studies were also amplified and sequenced. These are from *Blastocystis* sp. cultures from human and animal samples that were detected using PCR but not sequenced in previous studies. The animal samples were from chicken (n = 6), macaques (n = 3), and pigs (n = 11) collected for the study of Rivera [19] and from a dog (n = 1) collected for the study of Belleza et al. [28]. The stored DNA extracts of human samples were from the previous studies of Rivera [19], Santos and Rivera [29], and Adao et al. [21]. Those from the study of Rivera [19] were asymptomatic residents from the provinces of Batangas (n = 3) and Rizal (n = 6). Those from the study of Santos and Rivera [29] were from asymptomatic residents from the town of San Isidro, Rizal (n = 13). Furthermore, those from the study of Adao et al. [21] were from asymptomatic residents of backyard farmers from the province of Laguna (n = 3). Additionally, there were several other stored DNA extracts of *Blastocystis* sp. from unpublished studies where *Blastocystis* sp. was detected by PCR but not sequenced. These include samples from an asymptomatic zookeeper from the Manila Zoo (n = 1), asymptomatic residents of the informal settlement in BASECO compound in the City of Manila (n = 11), patients with gastrointestinal symptoms from the Philippine General Hospital (n = 4), and patients with gastrointestinal symptoms from the Philippine Heart Center (n = 2). All new *Blastocystis* sp. sequences from humans reported in this study are from stored DNA extracts. All patients gave approved consent forms for providing stool samples when these were collected in previous studies.

The barcoding regions of these *Blastocystis* sp. DNA extracts were amplified and sequenced similar to the methods stated above. In the case of samples P1 and P8 from pigs, the entire SSU rRNA gene was sequenced using the protocol and primers of Yoshikawa et al. [30] because the barcode sequences were only 91%–96% similar to reference sequences. This was conducted to confirm if these were new STs or errors encountered during amplification and sequencing because of the presence of contaminations.

2.7. Creating a database of all Blastocystis sp. ST-host data from the Philippines

A database of all *Blastocystis* sp. SSU rRNA gene sequences from the Philippines was created by combining the data from the new sequences collected in this study and from previously published sequences (Table 1). Published *Blastocystis* SSU rRNA gene sequences from studies in the Philippines were retrieved from GenBank. These include the studies of Rivera [19], Adao et al. [20], Adao et al. [21], Banaticla and Rivera [22], Evidor and Rivera [24], and Adao et al. [31]. All new sequences were also deposited in GenBank. Creating the database followed random non-probability sampling to cover as many types of hosts/sources as possible from those that can be acquired either through new samples or downloading sequences from Genbank. The entire set of *Blastocystis* SSU rRNA gene sequence data from the Philippines was used for the analysis of ST–host distribution. Meanwhile, subsets of this database were used for ST–allele distribution, genetic variability analyses, and comparison of populations using F_{ST}. A flowchart of the methods is shown in Fig. 1 and the subsets of data used for the different analyses are shown in Fig. 2. Prevalence of *Blastocystis* in farm animal and toad stool samples, cockroach guts, and water samples was expressed as percentages. Likewise, STs present per host or source were also expressed in percentages of the total number of sequences in that particular host or source.

2.8. ST–host distribution

The ST–host distribution of *Blastocystis* in the Philippines was analyzed by combining the data from *Blastocystis* SSU rRNA gene sequences and their respective hosts mentioned above and additional data from human and dog samples where *Blastocystis* were subtyped using the STS primers [28]. These were presented in Table 2 with summary of total number of STs and samples per host.

2.9. ST–allele distribution

A subset of all ST1–ST7 *Blastocystis* SSU rRNA gene sequences were uploaded onto the *Blastocystis* sp. PubMLST website (https://pubmlst.org/blastocystis/) to verify the STs and determine the alleles. For ST7, the closest matches were indicated if no exact matches

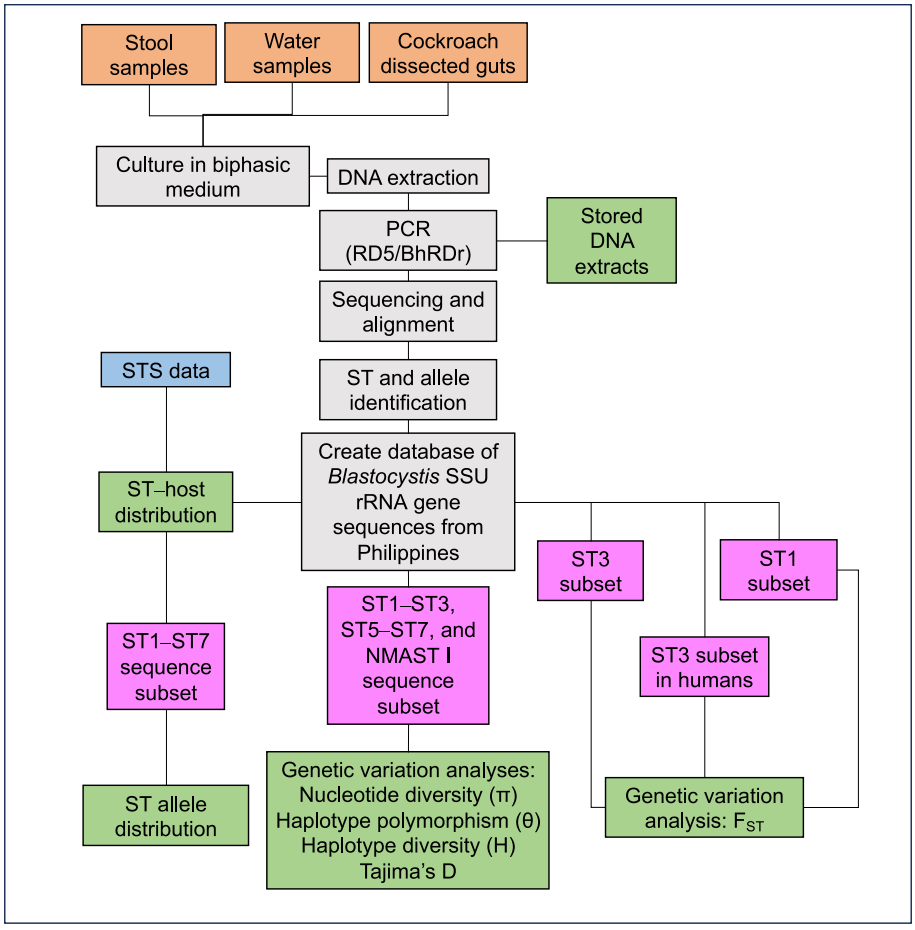

Fig. 1. Flowchart of methods and subsets of data used in this study.

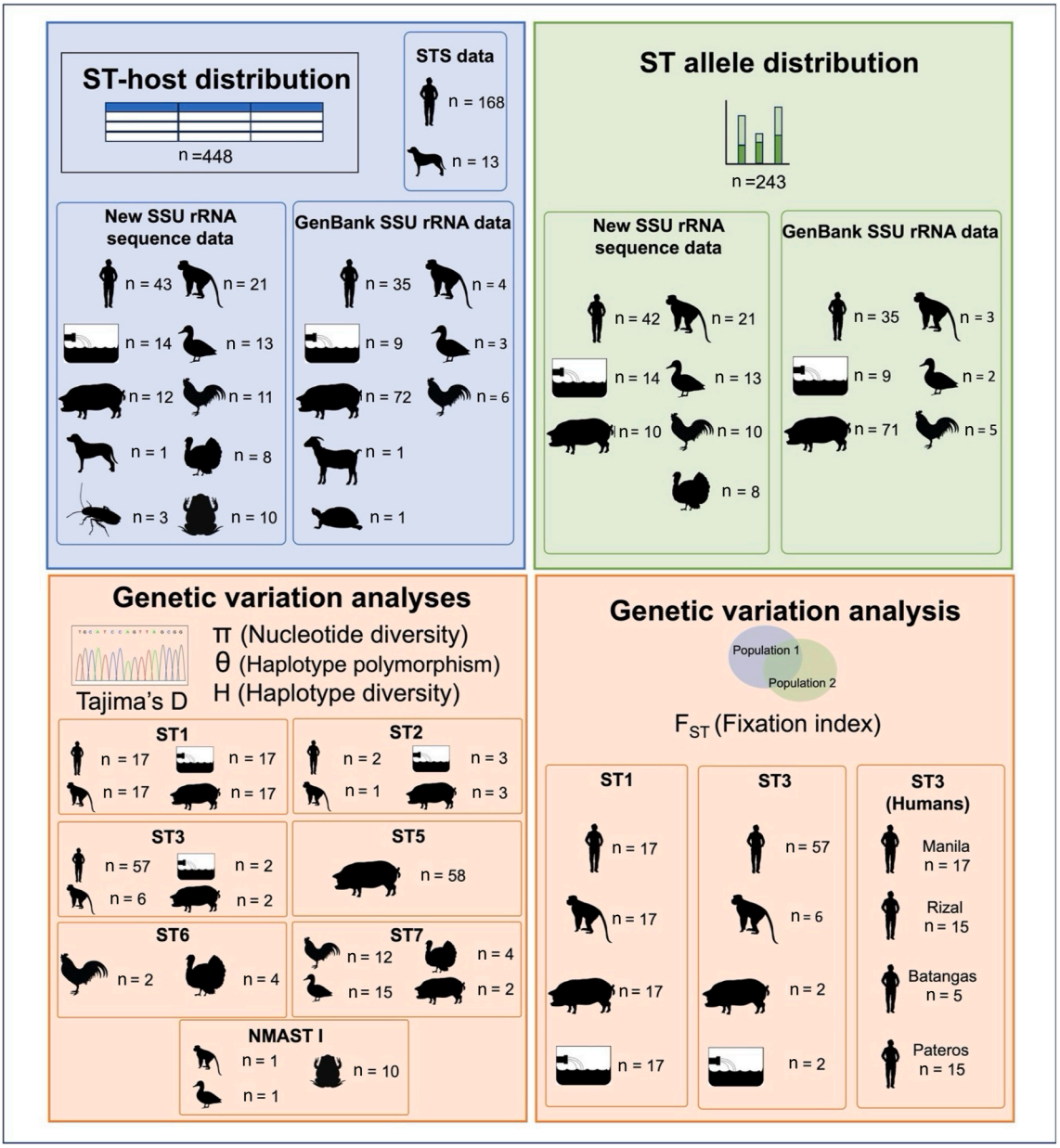

Fig. 2. Subsets of *Blastocystis* ST data used for analyses of ST–host distribution, ST–allele distribution, genetic variability analyses, and comparison of populations using fixation index (F_{ST}). Number of samples from each host or source used for the different analyses are indicated.

were found. The ST alleles identified in ST1–ST7 from humans, macaques, pigs, water samples, and poultry were summarized in Fig. 3.

2.10. Genetic variability analysis

Another subset of the *Blastocystis* SSU rRNA gene data containing all ST1–ST3, ST5–ST7 and NMAST I sequences was used for the genetic variability analyses. These were the sets of ST and NMAST sequences with sufficient number of sequences for genetic variability analyses using the program DNASP v5 [32]. These analyses included nucleotide diversity (π) or the average proportion of nucleotide differences between all possible pairs of samples; haplotype diversity (H) or probability of picking two different haplotypes in a population and nucleotide polymorphism (θ) or proportion of nucleotide sites that are expected to be polymorphic in any suitable sample from this region of the genome; and Tajima's D or test of neutrality to determine if the population is evolving randomly or under the influence of a nonrandom process [33]. The formulas used by DNASP v5 for calculating π, H, and θ are in Rozas [34] while the formulas used for calculating Tajima's D are similar to those used in Tajima et al. [35]. The sequences were grouped by ST, and the

Table 2
Blastocystis sp. ST diversity and distribution in various hosts/sources from the Philippines determined using both STS primers and SSU rRNA gene sequencing.

Host/Source	ST1	ST2	ST3	ST4	ST5	ST6	ST7	ST14	NMAST I	NMAST VI	Mixed	Total
Human	61 (24.80%)	8 (3.25%)	138 (56.10%)	30 (12.20%)	8 (3.25%)	0	0	0	0	0	1	246
Pig	17 (20.24%)	3 (3.57%)	3 (3.57%)	0	56 (66.67%)	0	2 (2.38%)	0	0	0	3 (3.57%)	84
Chicken	0	0	0	0	0	2 (11.76%)	13 (76.47%)	0	0	0	2 (11.76%)	17
Duck	0	0	0	0	0	0	15 (93.75%)	0	1 (6.25%)	0	0	16
Turkey	0	0	0	0	0	4 (50.00%)	4 (50.00%)	0	0	0	0	8
Macaque	17 (68.00%)	1 (4.00%)	6 (24.00%)	0	0	0	0	0	1 (4.00%)	0	0	25
Water	17 (73.91%)	3 (13.04%)	2 (8.70%)	1 (4.35%)	0	0	0	0	0	0	0	23
Dog	2 (14.29%)	2 (14.29%)	4 (28.57%)	3 21.43%)	3 (21.43%)	0	0	0	0	0	0	14
Goat	0	0	0	0	0	0	0	1 (100%)	0	0	0	1
Box turtle	0	0	0	0	0	0	0	0	1 (100%)	0	0	1
Cockroach	0	0	0	0	0	0	0	0	0	1 (33.33%)	2 (66.6%)	3
Toad	0	0	0	0	0	0	0	0	10 (100%)	0	0	10
Total	114	17	153	34	67	6	34	1	14	1	8	448

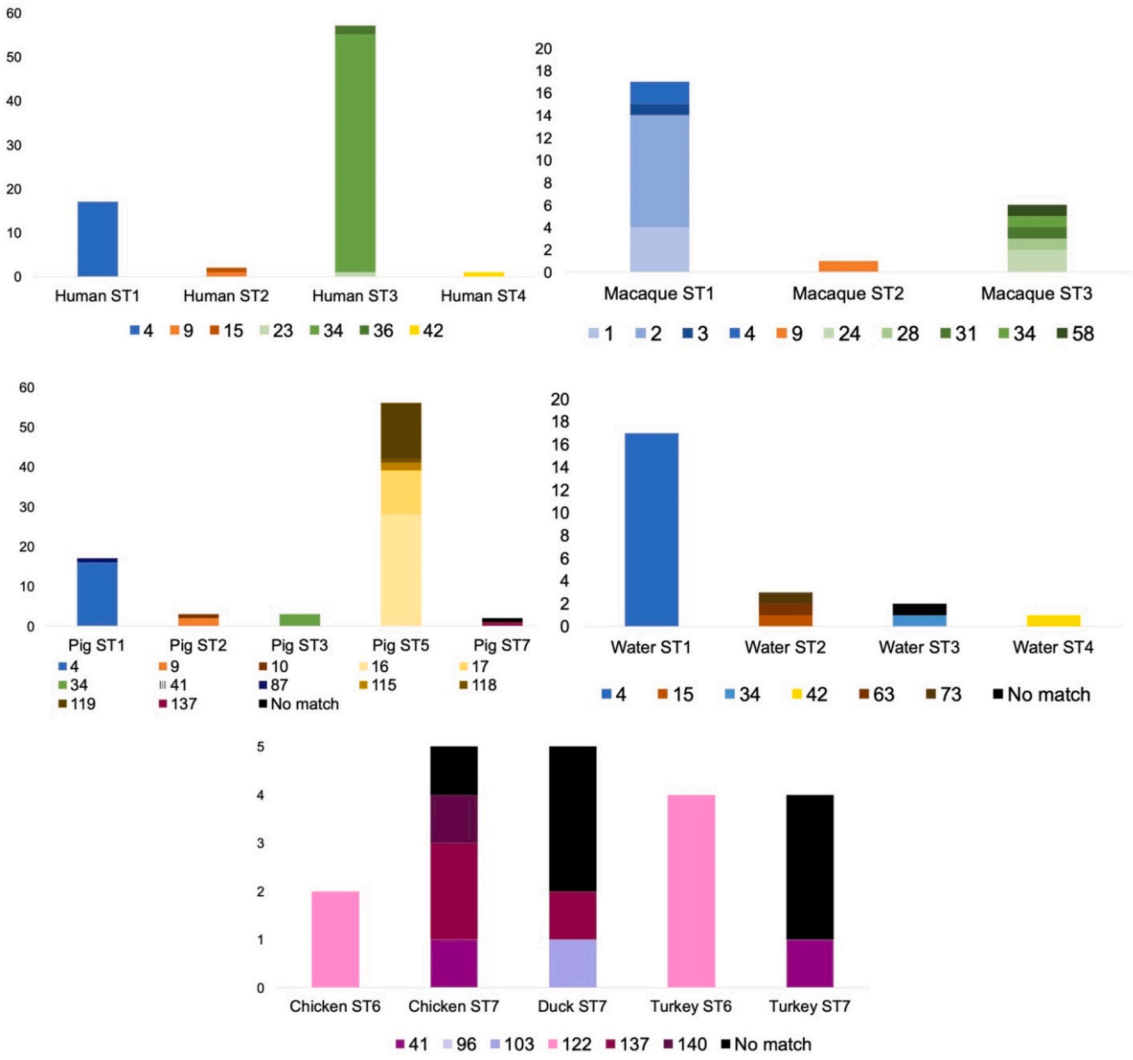

Fig. 3. Distribution of *Blastocystis* alleles identified in humans, pigs, macaques, water samples, and poultry from the Philippines. The color-coded numbers below each chart indicate the allele number for the particular ST. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

values for π% and H were used to determine their classifications based on whether these values are large (π% > 0.5%; H > 0.5) or small (π% < 0.5%; H < 0.5). These classifications are recent population bottleneck or founder effect (π% < 0.5%; H < 0.5), population bottleneck followed by recent expansion or rapid population growth (π% < 0.5%; H > 0.5), divergence between geographically subdivided populations (π% > 0.5%; H < 0.5), and large stable population with long evolutionary history or secondary contact between differentiated lineages (π% > 0.5%; H > 0.5) [36]. These classifications are used for marine fishes but are also applicable to microbes such as viruses [37–39] and *Plasmodium* [40,41].

2.11. Genetic differentiation of Blastocystis sp. ST1 and ST3 populations

Subsets of ST1 and ST3 sequences were used to compute the F_{ST}, which measures genetic variation among subpopulations from major groups of hosts/sources (humans, pigs, poultry, macaques, and water), as well as between and among ST3 human populations. Common values of F_{ST} for expressing genetic differentiation were followed in this study: small (0–0.05), moderate (0.05–0.15), great (0.15–0.25), and huge (>0.25) [33]. The formulas used by DNASP v5 for calculating F_{ST} are in Hudson et al. [42]. ST1 sequences were grouped by host/source: humans, macaques, pigs, and water. ST3 sequences were grouped as human or nonhuman, and all ST3 sequences from humans were grouped by location: the City of Manila, Municipality of Pateros, and the provinces of Rizal and Batangas. The former two areas are part of the National Capital Region of the Philippines while Rizal and Batangas are nearby provinces.

3. Results

3.1. Prevalence in the animal and water samples

Poultry and goat stool samples were taken from animals from a farm in Tanay, Rizal. A total of 4 of 13 (30.77%) chicken samples were positive for *Blastocystis* sp. Meanwhile, 13 of 19 (68.42%) ducks and 8 of 23 (34.78%) turkeys were culture positive for the gut protozoan. *Blastocystis* sp. was not detected in goats. Only 1 of 4 chicken sequences was ST6, and the rest were ST7. All duck samples were ST7. Four turkey samples were ST6, and the other four were ST7. There were also 10 of 64 (15.63%) toad stool samples from Quezon City positive for *Blastocystis*, and all were NMAST I. A pig stool sample from Laguna and 1 of 5 (20%) chicken stool samples from Quezon City were also positive for *Blastocystis* sp., with ST5 and ST7, respectively. *Blastocystis* sp. sequences were obtained from 18 of 63 (28.57%) macaques from the Parks and Wildlife Bureau (PAWB), 13 of 51 water samples (25.49%), and 3 of 127 (2.36%) cockroaches. The macaque samples consisted of ST1 (83.33%), ST3 (11.11%), and NMAST I (5.56%). *Blastocystis* sp. was found in 14 of 51 water samples (27.45%). These were identified as ST1 (71.43%), ST2 (7.14%), ST3 (14.29%), and ST4 (7.14%). There were 3 out of 127 (2.36%) cockroaches that were positive for *Blastocystis*. One cockroach sample was identified as NMAST VI, and the other two had overlapping chromatograms with 90% and 89% identity to *Blastocystis* sp., indicating mixed cultures. A summary of the prevalence and identified STs of all *Blastocystis* sp. from stool samples, water samples, and cockroach gut obtained for this study is presented in Table 3. In addition, sequences were obtained from stored DNA sequences from humans (n = 43), pigs (n = 12), macaques (n = 3), chickens (n = 7), and a dog (n = 1). Two stored DNA extracts from pigs (P1 and P8) had barcode sequences that were only 95% similar to GenBank sequences. P1 was verified to be ST2 and P8 as ST1 after full SSU rRNA gene sequencing. A summary of the STs identified from stored *Blastocystis* sp. DNA extracts is presented in Table 4.

3.2. ST–host distribution of Blastocystis sp. in samples from the Philippines

A total number of 267 partial 600-bp and full *Blastocystis* sp. SSU rRNA gene sequences were obtained for the database for ST and allele analysis (Table 1). These include 136 new sequences obtained in this study and stored in GenBank. For ST analysis, an additional 181 samples from the study by Belleza et al. [23] subtyped using STS primers were added, for a total of 448 samples with ST and host data. There were 8 STs (ST1-ST7 and ST14) and 2 NMASTs (NMASTs I and VI) identified so far. ST1–ST5 were found to be present in patients from the Philippines with ST3 (56.1%) as the most common in human samples. A similar pattern was found in dogs. ST1–ST3 and NMAST I were present in macaques with ST1 (68%) as the most commonly encountered. For pigs, ST1–ST3, ST5 and ST7 were found to be present with ST5 (66.67%) as the most common. ST6 and ST7 were commonly encountered in chicken and turkey while ST7 and NMAST I were present in ducks. ST7 was the most common in poultry ranging in prevalence from 50 to 93.75%. ST1–ST4 were found in water samples with ST1 (73.91%) as the most commonly encountered. NMAST I (100%) was present in toads while NMAST VI (33.3%) was found in cockroach. The summary of the *Blastocystis* STs identified from different hosts or sources in the Philippines using both PCR and sequencing and STS primers and their percent counts are shown in Table 2.

Table 3
Blastocystis sp. prevalence and identified STs and NMASTs from stool sample, water samples, and cockroach guts obtained for this study.

Host/Source (n)	Sample type	Location	*Blastocystis* prevalence (%)	ST and NMASTs
Chicken (13)	Stool	Tanay, Rizal	4 (30.77%)	ST6 (1), ST7 (3)
Duck (19)	Stool	Tanay, Rizal	13 (68.42%)	ST7 (13)
Turkey (23)	Stool	Tanay, Rizal	8 (34.78%)	ST6 (4), ST7 (4)
Toad (64)	Stool	Quezon City	10 (15.63%)	NMAST I (10)
Pig (1)	Stool	Laguna	1 (100%)	ST5
Chicken (5)	Stool	Quezon City	1 (20%)	ST7
Macaque (63)	Stool	Parks and Wildlife Bureau (PAWB), Quezon City	18 (28.57%)	ST1 (15), ST3 (2), NMAST I (1)
Water (14)	Creek water	Quezon City	2 (14.29%)	ST1 (2)
Water (2)	Flood water	Quezon City	1 (50%)	ST1 (1)
Water (2)	Wastewater	Quezon City	0	N/A
Water (1)	River water	Manila	1 (100%)	ST1 (1)
Water (2)	Creek water	Manila	1 (50%)	ST1 (1)
Water (2)	River water	Marikina	0	N/A
Water (7)	River water	Muntinlupa	4 (57.14%)	ST1 (3), ST4 (1)
Water (2)	River water	Taguig	1 (50%)	ST1 (1)
Water (1)	Creek water	Taguig	0	N/A
Water (2)	Creek water	Pasay	0	N/A
Water (2)	Wastewater	Batangas	0	N/A
Water (3)	River water	Laguna	0	N/A
Water (2)	Lake water	Laguna	1 (50%)	ST3 (1)
Water (3)	Wastewater	Laguna	2 (66.67%)	ST1 (1), ST2 (1)
Water (1)	Creek water	Rizal	0	N/A
Water (4)	Creek water	Bulacan	1 (25%)	ST3 (1)
Water (1)	River water	Bulacan	0	N/A
Cockroach (127)	Gut	Quezon City	3 (2.36%)	NMAST VI (1), mixed (2)

Table 4
Blastocystis STs obtained from stored DNA extracts.

Host/Source (n)	Location	ST
Human, asymptomatic (19)	Rizal	ST1 (4), ST2 (2), ST3 (13)
Human, symptomatic (3)	Batangas	ST3 (3)
Human, asymptomatic (11)	BASECO, Manila	ST1 (2), ST3 (9)
Human, asymptomatic (1)	Manila Zoo	ST3 (1)
Human, asymptomatic (3)	Victoria, Laguna	ST3 (1)
Human, symptomatic (4)	Philippine General Hospital	ST3 (4)
Human, symptomatic (2)	Philippine Heart Center	ST3 (2)
Pig (9)	Batangas	ST2 (1), ST3 (1), ST5 (5), mixed (2)
Pig (3)	Victoria, Laguna	ST3 (1), ST5 (1), ST7 (1)
Macaque (3)	Rizal	ST3 (3)
Chicken (5)	Batangas	ST7 (5)
Dog (1)	Laguna	ST1 (1)

3.3. Subtype and allele distribution

Several alleles were identified for ST1, ST3, ST5, and ST7 (Fig. 3), and only one allele was identified for ST4 (allele 42) and ST6 (allele 122). There were 26 sequences that had no exact allele matches. These were ST3 from water (n = 1), ST7 from chickens (n = 8), ST7 from ducks (n = 13), ST7 from turkeys (n = 3), and ST7 from pigs (n = 1). NMAST I, NMAST VI, and ST14 are not included in the allele database. The most commonly encountered ST alleles were ST1 allele 4, ST3 allele 34, and ST2 allele 9. ST1 allele 4 was identified in humans, macaques, pigs, water, and dogs. ST3 allele 34 was found in all of the same sources, except for dogs. In general, more ST alleles were identified in animals compared to humans. ST3 and ST1 in humans were mostly allele 34 and allele 4, respectively. Meanwhile, there were 3–5 alleles encountered in the most common STs in animal samples. Five alleles each were identified in ST3 (alleles 24, 28, 31, 34 and 58) and four in ST1 (alleles 1, 2, 3, 4 and 31) in macaques. ST1 alleles 1 and 2 and ST3 allele 24 were the most common in macaques. There were also 5 alleles identified in *Blastocystis* ST5 from pigs. These were alleles 16, 17, 115, 118, and 119. Of the 5, ST5 allele 16 was the most commonly encountered. In poultry, there were 5 *Blastocystis* sp. ST7 alleles identified (alleles 41, 96, 103, 137, and 140). There were 24 ST7 sequences that had unknown or no exact matches in poultry.

Blastocystis sp. ST1, ST3, and ST4 alleles in water samples were the same as those identified in humans. For ST2, alleles 63 and 73 were unique to water samples while allele 15 was also found in human samples. Only one allele was identified for ST6 (allele 122) and ST4 (allele 42).

3.4. Genetic variability analysis

Nucleotide diversity (π), haplotype polymorphism (θ), haplotype diversity (H), and Tajima's D for ST1, ST2, ST3, ST5, ST6, ST7, and NMAST I are presented in Table 5. The interpretation of the results is also presented. NMAST I had the highest values for π% (6.011%), θ (0.09892), and H (0.897), and ST6 had the lowest values at 0. These values were also higher in ST2, ST5, and ST7 compared with ST1 and ST3. Notably, overall sequence analysis of the subsets of ST1, ST3, ST5, and NMAST I sequences used show signs of recent population expansions or influx of new alleles. Meanwhile, ST2 and ST7 sequence sets show neutrality based on Tajima's D.

3.5. Comparison of ST1 and ST3 populations using fixation index (F_{ST})

The F_{ST} values between *Blastocystis* sp. ST1 and ST3 populations are presented in Table 6. *Blastocystis* sp. ST1 populations from Philippine macaques had huge genetic differentiation ($F_{ST} > 0.25$) when compared with ST1 sequences from humans, pigs, and water. ST3 populations from humans also had huge genetic differentiation compared with nonhuman populations. Between human populations, ST3 genetic differentiation was small. Notably, those from patients in Batangas and Rizal had identical sequences ($F_{ST} = 0$).

4. Discussion

4.1. Blastocystis ST distribution in different hosts or sources

ST–host associations were mostly the same for other studies, particularly in Southeast Asia. ST3 was the most commonly encountered in humans, followed by ST1. The same pattern has been reported in most studies around the world [3], including Southeast Asia [43]. In these cases, ST3 typically comprises 45%–90% of *Blastocystis* sp. STs identified in humans, whether these be immunocompromised [44], symptomatic [45–48], or asymptomatic individuals [49–54]. However, there are instances where ST1 is the most commonly encountered in humans, followed by ST3. These have occurred in surveys conducted from Southeast Asia [55–58], Africa [59–62], and the Americas [15,63–66], as well as from Australia [67], Netherlands [68], and Iran [69]. Typically, ST1–ST9 are encountered in humans, most especially ST1–ST4 [3,70]. In the Philippines, ST1–ST5 have been detected using either PCR and sequencing or PCR using STS primers (Table 2). There were no reports of rarely encountered STs, most probably because the surveys

Table 5
Genetic variability measures nucleotide diversity (π), haplotype diversity (H), haplotype polymorphism (θ), and Tajima's D of *Blastocystis* sp. ST1–ST3, ST5–ST7 and NMAST I sequences. Also included are the genetic variability measures for subsets of ST1 (humans, Philippine macaques, pigs, water samples) and ST3 (human and nonhuman sources; humans from City of Manila, Municipality of Pateros, Rizal province, and Batangas province).

Host/Subtype/ Source	N	π	π%	H	θ	Nucleotide and haplotype diversity	Interpretation	Tajima's D	Sig.	Interpretation
ST1 overall	68	0.00650	0.65%	0.617	0.02496	High π% and high H	Category 4 or large stable population with long evolutionary history	−2.4622	P < 0.01[a]	Reject null hypothesis of neutrality; Population expansion
Human	17	0.00626	0.626%	0.875	0.00745	High π% and high H	Category 4 or large stable population with long evolutionary history	−0.6432	P > 0.1	Accept null hypothesis of neutrality
Pig	17	0.00708	0.708%	0.728	0.01348	High π% and high H	Category 4 or large stable population with long evolutionary history	1.87685	P < 0.05[a]	Reject null hypothesis of neutrality; Population contraction
Macaque	17	0.00338	0.338%	0.618	0.0036	Low π% and high H	Category 2 or population bottleneck followed by rapid population growth	0.0138	P > 0.1	Accept null hypothesis of neutrality
Water	17	0.00833	0.833%	0.228	0.01621	High π% and low H	Category 3 or divergence between geographically subdivided populations	1.76809	P < 0.05[a]	Reject null hypothesis of neutrality; Population contraction
ST2 overall	9	0.02750	2.75%	0.944	0.03797	High π% and high H	Category 4 or large stable population with long evolutionary history	−1.39843	P > 0.10	Accept null hypothesis of neutrality
ST3 overall[b]	67	0.00366	0.366%	0.466	0.01351	Low π% and low H	Category 1 or recent population bottleneck or founder effect	−2.34432	P < 0.01[a]	Reject null hypothesis of neutrality; Population expansion
Human	57[a]	0.00286	0.286%	0.407	0.01246	Low π% and low H	Category 1 or recent population bottleneck or founder effect	−2.50695	P < 0.001[a]	Recent population bottleneck but heading towards expansion
Nonhuman	10	0.00558	0.558%	0.711	0.00489	High π% and high H	Category 4 or large stable population with long evolutionary history	0.25304	P > 0.1	Accept null hypothesis of neutrality
Human (Manila)	17	0.00179	0.179%	0.419	0.0034	Low π% and low H	Category 1 or recent population bottleneck or founder effect	−1.65847	0.01 > P > 0.5	Accept null hypothesis of neutrality
Human (Rizal)	15	0	0	0	0	Zero π% H		N/A	N/A	
Human (Batangas)	5	0	0	0	0	Zero π% H		N/A	N/A	
Human (Pateros)	19	0.00671	0.671%	0.737	0.01315	High π% and High H	Category 4 or large stable population with long evolutionary history	−1.9194	P < 0.05[a]	Reject null hypothesis of neutrality; Population expansion
ST5 overall	58	0.01143	1.143%	0.704	0.04197	High π% and high H	Category 4 or large stable population with long evolutionary history	−2.51355	P < 0.001[a]	Reject null hypothesis of neutrality; Population expansion
ST6 overall	6	0	0	0	0	Zero π% H		N/A		
ST7 overall	33	0.01686	1.686%	0.767	0.03148	High π% and high H	Category 4 or large stable population with long evolutionary history	−1.70755	0.10 > P > 0.05	Accept null hypothesis of neutrality
NMAST I overall	12	0.06011	6.011%	0.897	0.09892	High π% and high H	Category 4 or large stable population with long evolutionary history	−1.79283	P < 0.05[a]	Reject null hypothesis of neutrality; Population expansion

[a] P-value is significantly different.
[b] The overall number of human samples includes a lone sample from the province of Laguna, which was not included in the analysis per province or city of the Philippines.

Table 6
Fixation index (F_{ST}) values between groups within *Blastocystis* sp. ST1 and ST3.

ST1		F_{ST}
Group 1	Group 2	
Human	Macaque	0.57870
Human	Pig	0.03271
Human	Water	0.04521
Macaque	Pig	0.53070
Macaque	Water	0.43838
Pig	Water	0.01904
ST3		
Human	Nonhuman	0.26033
Manila	Rizal	0.05357
Manila	Batangas	0.05357
Manila	Pateros	0.04608
Rizal	Batangas	0
Rizal	Pateros	0.04406
Batangas	Pateros	0.04406

were conducted in highly urbanized and semi-urbanized areas of the National Capital Region and its nearby provinces. By contrast, a survey in Da Nang City, Vietnam, showed the presence of ST8, ST10, and ST14 in patients [48], and ST10 and ST23 were encountered in residents of a rural village in Chiang Rai Province, Thailand [71]. Surveys in other parts of the Philippines, especially in rural areas or provinces occupied by indigenous groups, where there is more contact with domestic or wild animals, may show more STs carried by patients besides ST1–ST5.

Blastocystis STs identified in animals were also mostly those associated with their respective groups. ST1 was the most commonly encountered in macaques (nonhuman primates), but ST2 and ST3 were also identified. In this study, ST5 was the most commonly encountered in pigs, followed by ST1, ST2, ST3, and ST7. Meanwhile, ST7 and ST6 were the most commonly encountered in poultry. ST1–ST3 are the most commonly encountered *Blastocystis* STs in nonhuman primates in Southeast Asia [72–74], although reports from other parts of the world show that ST4, ST5, ST7, ST8, ST9, ST10, ST13, and ST15 are also present in this animal group [4,75]. Because data and samples from only one species of nonhuman primate were available for this study (*Macaca fascicularis*), it is also possible that other STs will be identified if samples are taken from the other primate species in the country, such as the Philippine tarsier (*Carlito syrichta*) and Philippine slow loris (*Nycticebus menagensis*) [76]. *Blastocystis* ST5 is the most commonly encountered *Blastocystis* ST in pigs in other parts of the world [10,53,56,74], and ST6 and ST7 are the most commonly encountered in birds, including poultry [2]. ST7, in particular, was also identified in pigs in this study, similar to the results of the study by Jinatham et al. [71] in Thailand. In addition, ST7 has been reported in a buffalo in the same study from Thailand [71], goats from Malaysia [77], and more commonly, in recent studies surveying humans from Thailand [71,78] and Vietnam [48]. This indicates that ST7 may have a wider host range than initially recorded besides birds. This may be due to the proximity of poultry and other bird species with livestock and people. The pigs with ST7 included in this study were housed in backyard farms [21] which typically also have other bird species in the farms such as chickens, ducks, and pigeons. This further shows that ST7 cross-contamination is possible between different host species.

There were few STs identified from dogs and goats. These were ST1–ST5 from dogs and ST14 from goats. The identified STs from dogs and goats are also similar to the STs identified from studies in Southeast Asia [73]. ST1–ST4, which are common in humans, have been reported in dogs from the Philippines [23] and goats from Malaysia [77]. In contrast, ST14 was not identified in humans in this study but it has been previously reported in schoolchildren from Senegal [79]. No *Blastocystis* cultures were observed out of the 11 goat stool samples collected. This is in contrast to previous reports of prevalence of up to 30.9% with ST1, ST3, ST6, and ST7 identified in goats [77].

There has been considerable interest recently in identifying *Blastocystis* sp. in poikilothermic animals or those that change their body temperatures with the environment such as insects, amphibians, reptiles, and fish. The identified *Blastocystis* sequences in these animals used to cluster separately with the previously identified 17 STs from mammalian and avian samples, and they are thus identified as NMASTs known as NMAST I to NMAST VIII [9,10]. However, these NMASTs cluster with mammalian and avian STs when 27 STs (ST1–ST17, ST21, ST23–ST31) are included in the phylogenetic tree [11]. Moreover, there are instances when mammalian and avian STs are reported in poikilothermic animals and NMASTs are found in mammalian and avian hosts [9,11,21,80]. In this study, NMAST I was identified in toads, box turtles, Philippine macaques, and ducks and NMAST VI in cockroaches. NMAST I has been previously reported in reptiles and frogs, and NMAST VI has been previously reported in cockroaches [9,10]. No *Blastocystis* STs associated with humans were found so far, unlike in other studies where ST2, ST3, and ST4 were identified in cockroaches [81–83]. This is the first report of NMAST I in Philippine macaques and the first report of *Blastocystis* sp. in cockroaches in the Philippines. The results of this study are additional evidence for the presence of NMASTs in mammalian and avian hosts and support the idea of NMASTs and STs having wider host ranges than previously established.

Blastocystis ST1–ST4 were identified in water samples. ST1 and ST2 were previously identified in wastewater samples from different facilities in the Philippines [22]. In this study, *Blastocystis* ST1 was identified in river water, creek water, lake water, and floodwater. Meanwhile, ST2 was identified in lake water; ST3, in creek water; and ST4, in river water. Contaminated water is a possible source of *Blastocystis* contamination, with similar STs identified in both residents and water samples in the area [71,78,84,85]. In these studies, ST1 and ST3, which are the most common in humans, were identified in both nearby water sources and residents [78,84]. However,

rare human STs such as ST10 and ST23 have also been identified in both water storage containers and residents of a rural village in Thailand [71]. Tap water is also a possible source of *Blastocystis* ST3 [86]. Moreover, *Blastocystis* ST3 has been identified in vegetables sold in wet markets [87] and ST1, ST3, ST7, ST23, and ST26 in soil [71]. This shows that *Blastocystis* transmission via contaminated water, food, and soil may play a bigger role in cross-transmission of *Blastocystis* between different host species.

4.2. *Blastocystis* ST alleles in ST1–ST7

There were more ST alleles identified in the most common STs in animals compared to those found in humans. All ST1 alleles from humans were allele 4 while almost all the three ST3 alleles identified were allele 34. On the other hand, there were 3–5 alleles identified in ST5 from pigs, ST7 from poultry, and ST1 and ST3 from Philippine macaques in almost equal distribution. ST3 allele 34 is the most commonly ST3 allele in humans from studies in Iran [88], Colombia [89] and Italy [90]. In contrast, ST3 allele 38 is the most commonly encountered in South America [70] and in a study in children from Colombia [91]. On the other hand, ST1 allele 4 was also the most commonly encountered in humans as well as allele 2 in these studies [70,88,89,91]. In these studies, there were also 3–9 ST3 alleles identified in comparison to only 3 in this study and 2–6 ST1 alleles compared to just one in this study. A more extensive sampling from other parts of the country may reveal more ST1 and ST3 alleles or even other less common STs in human samples. A homogenous population of *Blastocystis* sp. ST1 and ST3 is observed in humans residing in the National Capital Region and its nearby provinces.

ST5 allele 16 was the most commonly encountered in pigs and ST allele 2 was the most commonly encountered in macaques. In comparison, ST5 allele 115 was the most commonly encountered in pigs from Italy [92] and ST1 allele 2 was the most commonly identified in long-tailed macaques from Thailand [72]. Not much data on allele distribution is available for animals and water samples. Thus, data from this study may be relevant in comparing the ST alleles found in these sources in future studies.

4.3. Genetic variability analyses

Genetic variability measures π, H, θ, and Tajima's D have been used to analyze *Blastocystis* sp. intrasubtype variations from humans [14,16,17,88,90] and nonhuman primates [16]. The values for π range from 0.00261 to 0.031 for ST1, 0.0001 to 0.972 for ST2, and 0.0008 to 0.628 for ST3 in these studies. Meanwhile, values for θ range from 0.0298 to 0.905 for ST2, 0.0238 to 0.972 for ST2, and 0.0054 to 0.628 for ST3. The values for π and θ for ST1, ST2, and ST3 computed in this study are all within range of these values except for value of θ for ST1, which was lower compared to those from other studies. This could be due to the presence of mostly similar ST1 allele 4 sequences which lowered the expected number of nucleotide positions that are expected to be polymorphic. Values for ST6 π were also previously computed for *Blastocystis* ST6 sequences obtained from humans from Italy [90]. In this study, the value for π for ST6 was 0 because all sequences were identical. It is possible that ST6 has very low nucleotide diversity for the sequences obtained in this study since there is also only one ST6 allele identified (allele 122). The values for π and θ for ST5, ST7 and NMAST I were also within the values of ST1–ST3 computed from these other studies. However, there is not much data from previous studies for these STs. These results may be relevant in future studies of genetic variability analysis of different *Blastocystis* sp. STs found in animals.

Tajima's D has also been used in previous studies to show neutrality of populations or determine either population contraction or expansion [14,17]. Both studies showed negative Tajima's D values indicating recent population expansion much like the results for ST1, ST3, ST5, ST7, and NMAST I. These genetic variability measures show that there is considerable intrasubtype diversity for these STs.

4.4. Fixation index (F_{ST}) to determine differentiation between ST1 and ST3 populations

Results show that ST1 populations in humans, pigs, and water most probably belong to the same circulating population with small genetic differentiation (F_{ST} values from 0.01904 to 0.04847). On the other hand, ST3 populations from humans are genetically distinct from those from non-human sources with great genetic differentiation ($F_{ST} = 0.24384$) while ST3 from humans most probably belong to the same circulating population with no to small genetic differentiation (F_{ST} values from 0 to 0.04688). F_{ST} values have been utilized in the past to differentiate circulating populations of *Blastocystis* sp. ST1 to ST3 between children and adults [17] or between patients with irritable bowel syndrome and asymptomatic patients [14]. It has also been used to compare populations of ST1 and ST2 between populations of howler monkeys and between populations found in howler monkeys and with humans that live nearby [16]. In this study, moderate differentiation was observed between ST1 populations in howler monkeys and humans while great differentiation was observed in ST2 populations.

4.5. *Blastocystis* intrasubtype diversity and population similarities

Blastocystis sp. intrasubtype diversity was determined by analyzing available data from number of alleles per ST and values of π, H, θ, and significant values of Tajima's D. Moreover, F_{ST} data was also used for ST1 and ST3. There were four observed patterns: selective sweep or expansion after a bottleneck and possibly reaching a stable large population (ST1 overall, ST5, and NMAST I), stable large population possibly headed to a population contraction (ST1 in pigs), recent population bottleneck event heading toward expansion (ST3 overall), and no variations (ST6 overall and ST3 in humans in Batangas and Rizal). In the case of ST1 and ST3, the added data from FST analysis supported the differentiation between populations.

The first pattern was observed in ST1, ST5, and NMAST I. These were characterized by a negative Tajima's D, >0.50% π, and

>0.5H. These populations were also characterized by a dominant allele with moderate counts of several other alleles. These indicate a possible large stable population (category 4) reached after a recent expansion from a bottleneck effect. In the case of ST1, this means that newer ST1 alleles were possibly introduced from cross-infections of ST1 between different hosts. ST1 was found in five different sources in this study, particularly ST1 allele 4 which fits its generalist profile [16]. ST1 is commonly found in various animal hosts, including humans [1,3,4], as well as in water samples [22,84,85] and is known to be possibly transferred via water to humans or animals [78,84]. This data together with F_{ST} values give supporting evidence of the same circulating populations of ST1 between pigs and humans possibly transferred via a waterborne route. By contrast, the macaque ST1 population was genetically distinct from these other ST1 populations. This indicates that proximity of livestock with humans and probably water contamination can contribute to cross-contamination between these hosts. The macaques sampled were housed in individual cages in a zoo facility, which hinders transfer between other hosts.

More data are needed in determining *Blastocystis* ST5 and NMAST I population differentiation. ST5 is known to be found in pigs and NMAST I in poikilothermic animals but have also been reported in other hosts [21,93], so introduction of new alleles through cross-infection from other host species is still possible. By contrast, the opposite is observed in ST1 in pigs compared with ST1 overall with a contracting population (Tajima's D = 1.87685). This may be due to *Blastocystis* ST1 in pigs being replaced by more new alleles from other STs from other pigs. However, more evidence is needed to confirm this.

Blastocystis ST3, particularly allele 34, was common in humans in this study but was also found in water samples, Philippine macaques, and pigs. Results indicate that the human ST3 population is so far not genetically similar to those from nonhuman sources in contrast to ST1. However, the data show overall recent expansion from a bottleneck effect, which could be due to the influx of new alleles in the sampled human populations and nonhuman sources. New *Blastocystis* ST3 alleles are also possibly introduced in humans in certain populations only in highly populated urban areas (Manila and Pateros) compared with those from the more rural areas (Rizal and Batangas). It is notable that the same circulating population was found in humans from Rizal and Batangas with zero nucleotide and haplotype diversity even when sampled almost 10 years apart. This further indicates that new alleles are most probably introduced in the urban areas with higher population and more people traveling in and out of the city. More genetically distinct *Blastocystis* has been found before in populations that travel and interact more with other populations, such as working adults, compared with those in children who often stay in the same area [17].

Blastocystis sp. ST6 had no sequence variation ($\pi\% = 0$; $H = 0$) and only one allele (122). Meanwhile, there were several unidentified alleles in ST7 but the population seems to have achieved a state of neutrality and is under category 4 (large stable population). In this case, *Blastocystis* sp. in poultry may be more diverse than expected but still comprised of the same circulating population within poultry populations in the areas sampled.

Blastocystis sp. ST2 and NMAST I were under category 4 or stable large population. However, there are only a few sequences of these STs, as well as ST4, to make proper conclusions based on their genetic variability. More samples are recommended to properly assess the genetic diversity and relatedness of the populations of these STs.

4.6. Limitations of the study

An extensive collection of *Blastocystis* sp. sequences was done but these were mostly taken from hosts and sources from the National Capital Region of the Philippines and its nearby provinces of Laguna, Rizal, Batangas, and Bulacan. Generalizations on ST–host distributions and ST–allele patterns can be made but these may still change given more data from other parts of the country. Moreover, smaller subsets of data were also used for ST–allele pattern analyses, genetic variability analyses, and fixation index to compare populations because STS data cannot be used for these analyses. ST allele analysis was only done for STs with available allele data (ST1–ST7) while genetic variability analyses was only done for certain ST populations with adequate number of sequences (ST1–ST3, ST5–ST7, and NMAST I). In the case of F_{ST}, these types of analyses were also limited to ST1 and ST3 because these were the only data subsets that had 4 or more sequences from each of the groups based on hosts or sources. DnaSP v 5 will only compute F_{ST} if there are 4 or more sequences from each group representing a population. For ST3, all sequences from nonhuman hosts or sources were compiled together to have more than 4 sequences. Additional samples from macaques, pigs, and water samples can produce more groups for analysis. Moreover, comparing population similarities using F_{ST} was based on the assumption that specific populations of *Blastocystis* sp. were more likely transferred between members of the same host species (e.g., human to human transmission) compared to different host species. Thus, it is similar to finding the same ST and alleles of *Blastocystis* sp. between two different host species which can lead to evidence of possible cross-transmission. In this case, low values of F_{ST} can indicate that the same circulating population of *Blastocystis* sp. can be found between two different hosts or sources.

5. Conclusion

Blastocystis sp. from the Philippines mostly follows the same ST–host patterns from other parts of the world, although some notable ST–host associations were noted. In general, more ST alleles were present in animals compared to humans. *Blastocystis* sp. populations of ST1, ST3, ST5, and NMAST I are undergoing population expansion possibly from addition of new alleles from outside sources while ST7 has a possible high allele diversity but remains genetically similar within the populations of poultry and pigs sampled. Genetic variability analyses also show stability of populations depending on the ST or NMAST. Analyzing *Blastocystis* sp. by populations can show certain patterns of transmission. In particular, ST1 populations in humans, pigs, and wastewater were found to be genetically similar indicating cross-infections between humans and livestock animals possibly through a waterborne route. Conversely, ST3 in humans is so far distinct from those found in nonhuman sources. Moreover, *Blastocystis* ST3 has diverse populations in humans living in

urban areas compared with those living closer to rural areas, which have identical ST3 sequences.

The data presented shows that *Blastocystis* sp. is highly diverse in the Philippines and that certain patterns of host transmission can be determined using combinations of allele diversity, genetic variability analyses, and population similarity analysis using F_{ST}. These types of analyses can also be done by ST assuming that the same circulating populations of these *Blastocystis* sp. STs are commonly shared between host species. However, these patterns were only observed for available sequences for *Blastocystis* sp. obtained from hosts and sources from the National Capital Region and nearby provinces of Laguna, Batangas, Rizal, and Bulacan. Adding more samples of *Blastocystis* sp. from indigenous groups living closely with domestic and wild animals and more samples from other parts of the country farther from the National Capital Region can show more patterns of ST-specific population transmission or even more ST–host associations or more unique ST–alleles. It is recommended that similar analyses be done in other parts of the country to add more sequences to the present database and to confirm if some generalized patterns – such as similar circulating ST1 and ST3 populations – will still be observed. This would also give the opportunity to observe if there are other circulating sub-populations of *Blastocystis* STs (e.g., ST2 and ST4) transferred between different hosts or sources.

CRediT authorship contribution statement

Davin Edric V. Adao: Writing – review & editing, Writing – original draft, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Windell L. Rivera:** Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Software, Resources, Project administration, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgement

This study was supported financially by a research grant from the Office of the Chancellor of the University of the Philippines Diliman through the Office of Vice Chancellor for Research and Development.

References

[1] M.A. Alfellani, A.S. Jacob, N.O. Perea, R.C. Krecek, D. Taner-Mulla, J.J. Verweij, B. Levecke, E. Tannich, C.G. Clark, C.R. Stensvold, Diversity and distribution of *Blastocystis* sp. subtypes in non-human primates, Parasitology 140 (2013) 966–971.
[2] C.R. Stensvold, M.A. Alfellani, S. Nørskov-Lauritsen, K. Prip, E.L. Victory, C. Maddox, H. V Nielsen, C.G. Clark, Subtype distribution of *Blastocystis* isolates from synanthropic and zoo animals and identification of a new subtype, Int. J. Parasitol. 39 (2009) 473–479.
[3] M.A. Alfellani, C.R. Stensvold, A. Vidal-Lapiedra, E.S.U. Onuoha, A.F. Fagbenro-Beyioku, C.G. Clark, Variable geographic distribution of *Blastocystis* subtypes and its potential implications, Acta Trop. 126 (2013) 11–18.
[4] M.A. Alfellani, D. Taner-Mulla, A.S. Jacob, C.A. Imeede, H. Yoshikawa, C.R. Stensvold, C.G. Clark, Genetic diversity of *Blastocystis* in livestock and zoo animals, Protist 164 (2013) 497–509.
[5] J.G. Maloney, M. Santin, Mind the gap: new full-length sequences of *Blastocystis* subtypes generated via Oxford Nanopore Minion sequencing allow for comparisons between full-length and partial sequences of the small subunit of the ribosomal RNA gene, Microorganisms 9 (2021) 997.
[6] J.G. Maloney, Y. Jang, A. Molokin, N.S. George, M. Santin, Wide genetic diversity of *Blastocystis* in white-tailed deer (*Odocoileus virginianus*) from Maryland, USA, Microorganisms 9 (2021) 1343.
[7] A. Higuera, G. Herrera, P. Jimenez, D. García-Corredor, M. Pulido-Medellín, D.M. Bulla-Castañeda, J.C. Pinilla, D.A. Moreno-Pérez, J.G. Maloney, M. Santín, J. D. Ramírez, Identification of multiple *Blastocystis* subtypes in domestic animals from Colombia using amplicon-based next generation sequencing, Front. Vet. Sci. 8 (2021) 932.
[8] J.G. Maloney, A. Molokin, R. Seguí, P. Maravilla, F. Martínez-Hernández, G. Villalobos, A.D. Tsaousis, E. Gentekaki, C. Muñoz-Antolí, D.R. Klisiowicz, Identification and molecular characterization of four new *Blastocystis* subtypes designated ST35-ST38, Microorganisms 11 (2022) 46.
[9] A. Cian, D. El Safadi, M. Osman, R. Moriniere, N. Gantois, S. Benamrouz-Vanneste, P. Delgado-Viscogliosi, K. Guyot, L.-L. Li, S. Monchy, Molecular epidemiology of *Blastocystis* sp. in various animal groups from two French zoos and evaluation of potential zoonotic risk, PLoS One 12 (2017) e0169659.
[10] H. Yoshikawa, Y. Koyama, E. Tsuchiya, K. Takami, *Blastocystis* phylogeny among various isolates from humans to insects, Parasitol. Int. 65 (2016) 750–759.
[11] V.Y.B. Meclat, K.S.B. Ancheta, D.E. V Adao, W.L. Rivera, Phylogenetic relationship of nonmammalian and avian *Blastocystis* isolates and conventional subtypes, J. Parasit. Dis. 47 (2023) 192–197.
[12] S.M. Scicluna, B. Tawari, C.G. Clark, DNA barcoding of *Blastocystis*, Protist 157 (2006) 77–85.
[13] C.R. Stensvold, M. Alfellani, C.G. Clark, Levels of genetic diversity vary dramatically between *Blastocystis* subtypes, Infect. Genet. Evol. 12 (2012) 263–273.
[14] G.-B. Vargas-Sanchez, M. Romero-Valdovinos, C. Ramirez-Guerrero, I. Vargas-Hernandez, M.E. Ramirez-Miranda, J. Martinez-Ocaña, A. Valadez, C. Ximenez, E. Lopez-Escamilla, M.E. Hernandez-Campos, *Blastocystis* isolates from patients with irritable bowel syndrome and from asymptomatic carriers exhibit similar parasitological loads, but significantly different generation times and genetic variability across multiple subtypes, PLoS One 10 (2015) e0124006.
[15] G. Villalobos, G.E. Orozco-Mosqueda, M. Lopez-Perez, E. Lopez-Escamilla, A. Córdoba-Aguilar, L. Rangel-Gamboa, A. Olivo-Diaz, M. Romero-Valdovinos, P. Maravilla, F. Martinez-Hernandez, Suitability of internal transcribed spacers (ITS) as markers for the population genetic structure of *Blastocystis* spp, Parasit. Vectors 7 (2014), https://doi.org/10.1186/s13071-014-0461-2.
[16] C. Villanueva-Garcia, E.J. Gordillo-Chavez, E. Lopez-Escamilla, E. Rendon-Franco, C.I. Muñoz-Garcia, L. Gama, W.A. Martinez-Flores, N. Gonzalez-Rodriguez, M. Romero-Valdovinos, H. Diaz-Lopez, Clarifying the cryptic host specificity of *Blastocystis* spp. isolates from *Alouatta palliata* and *A. pigra* howler monkeys, PLoS One 12 (2017) e0169637.
[17] I. Villegas-Gómez, F. Martínez-Hernández, A. Urrea-Quezada, M. González-Díaz, M. Durazo, J. Hernández, G.E. Orozco-Mosqueda, G. Villalobos, P. Maravilla, O. Valenzuela, Comparison of the genetic variability of *Blastocystis* subtypes between human carriers from two contrasting climatic regions of México, Infect. Genet. Evol. 44 (2016) 334–340.
[18] C. Noël, C. Peyronnet, D. Gerbod, V.P. Edgcomb, P. Delgado-Viscogliosi, M.L. Sogin, M. Capron, E. Viscogliosi, L. Zenner, Phylogenetic analysis of *Blastocystis* isolates from different hosts based on the comparison of small-subunit rRNA gene sequences, Mol. Biochem. Parasitol. 126 (2003) 119–124.
[19] W.L. Rivera, Phylogenetic analysis of *Blastocystis* isolates from animal and human hosts in the Philippines, Vet. Parasitol. 156 (2008) 178–182.

[20] D.E.V. Adao, A.O. Dela Serna, M.L.B. Belleza, N.R. Bolo, W.L. Rivera, Subtype analysis of *Blastocystis* sp. isolates from asymptomatic individuals in an urban community in the Philippines, Ann. Parasitol. 62 (2016) 193–200.
[21] D.E.V. Adao, R.J.T. Ducusin, M.A. Padilla, W.L. Rivera, Molecular characterization of *Blastocystis* isolates infecting farm animals in Victoria and Pila, Laguna, Philippines, Philipp, Agric. Sci. 99 (2016), 304–301.
[22] J.E.G. Banaticla, W.L. Rivera, Detection and subtype identification of *Blastocystis* isolates from wastewater samples in the Philippines, J. Water Health 9 (2011) 128–136.
[23] M.L.B. Belleza, J.C.B. Reyes, P.N. Tongol-Rivera, W.L. Rivera, Subtype analysis of *Blastocystis* sp. isolates from human and canine hosts in an urban community in the Philippines, Parasitol. Int. 65 (2016) 291–294.
[24] F.M.R. Evidor, W.L. Rivera, Genetic subtypes of *Blastocystis* sp. isolated from fecal materials in the large intestines of slaughtered swine, Philipp. J. Vet. Med. 53 (2016) 59–64.
[25] V. Zaman, G.C. Ng, K. Suresh, E.H. Yap, M. Singh, Isolation of *Blastocystis* from the cockroach (Dictyoptera: Blattidae), Parasitol. Res. 79 (1993) 73–74.
[26] W.L. Rivera, V.A. Ong, Development of loop-mediated isothermal amplification for rapid detection of *Entamoeba histolytica*, Asian Pac. J. Trop. Med. 6 (2013) 457–461.
[27] T.A. Hall, BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT, in: Nucleic Acids Symp. Ser., Oxford, 1999, pp. 95–98.
[28] M.L.B. Belleza, J.L.C. Cadacio, M.P. Borja, J.A.A. Solon, M.A. Padilla, P.N. Tongol-Rivera, W.L. Rivera, Epidemiologic study of *Blastocystis* infection in an urban community in the Philippines, J. Environ. Public Health. (2015) 894297.
[29] H.J. Santos, W.L. Rivera, Comparison of direct fecal smear microscopy, culture, and polymerase chain reaction for the detection of *Blastocystis* sp. in human stool samples, Asian Pac. J. Trop. Med. 6 (2013) 780–784.
[30] H. Yoshikawa, N. Abe, M. Iwasawa, S. Kitano, I. Nagano, Z. Wu, Y. Takahashi, Genomic analysis of *Blastocystis hominis* strains isolated from two long-term health care facilities, J. Clin. Microbiol. 38 (2000) 1324–1330.
[31] D.E.V. Adao, I.D.J. Ronquillo, Y.K.M. Dela Cruz, E.J.A. Pagoso, W.L. Rivera, Molecular characterization of *Giardia duodenalis* and *Blastocystis* sp. in livestock from animal farms in Bulacan, Philippines, Southeast Asian, J. Trop. Med. Public Health 50 (2019) 450–460.
[32] P. Librado, J. Rozas, DnaSP v5: a software for comprehensive analysis of DNA polymorphism data, Bioinformatics 25 (2009) 1451–1452.
[33] D.L. Hartl, A.G. Clark, A.G. Clark, Principles of Population Genetics, Sinauer Associates Sunderland, MA, 1997.
[34] J. Rozas, DNA sequence polymorphism analysis using DnaSP, in: D. Posada (Ed.), Bioinformatics for DNA Sequence Analysis. Methods in Molecular Biology, Humana Press, 2009, pp. 337–350.
[35] F. Tajima, R.R. Hudson, M. Slatkin, W.P. Maddison, J. Rozas, Statistical method for testing the neutral mutation hypothesis by DNA polymorphism, Genetics 123 (1989) 337–350.
[36] W.A.S. Grant, B.W. Bowen, Shallow population histories in deep evolutionary lineages of marine fishes: Insights from sardines and anchovies and lessons for conservation, J. Hered. 89 (1998) 415–426.
[37] M. Tsompana, J. Abad, M. Purugganan, J.W. Moyer, The molecular population genetics of the tomato spotted wilt virus (TSWV) genome, Mol. Ecol. 14 (2005) 53–66.
[38] T. Ogawa, Y. Tomitaka, A. Nakagawa, K. Ohshima, Genetic structure of a population of potato virus Y inducing potato tuber necrotic ringspot disease in Japan: comparison with North American and European populations, Virus Res. 131 (2008) 199–212.
[39] Y.P. Tian, J.L. Liu, C.L. Zhang, Y.Y. Liu, B. Wang, X.-D. Li, Z.K. Guo, J.P.T. Valkonen, Genetic diversity of potato virus Y infecting tobacco crops in China, Phytopathology 101 (2011) 377–387.
[40] U.W. Azlan, Y.L. Lau, M.Y. Fong, Genetic diversity and clustering of the rhoptry associated protein-1 of *Plasmodium knowlesi* from Peninsular Malaysia and Malaysian Borneo, Korean J. Parasitol. 60 (2022) 393.
[41] Y.L. Ng, Y.L. Lau, M.H.A. Hamid, J. Jelip, C.H. Ooi, R.N. Mudin, J.J. Jaimin, M.Y. Fong, Genetic polymorphism of the thrombospondin-related apical merozoite protein (TRAMP) of *Plasmodium knowlesi* in Malaysia, Parasitol. Res. 122 (2023) 195–200.
[42] R.R. Hudson, M. Slatkin, W.P. Maddison, Estimation of levels of gene flow from DNA sequence data, Genetics 132 (1992) 583–589.
[43] S. Popruk, D.E. V Adao, W.L. Rivera, Epidemiology and subtype distribution of *Blastocystis* in humans: a review, Infect. Genet. Evol. 95 (2021) 105085.
[44] T.C. Tan, S.C. Ong, K.G. Suresh, Genetic variability of *Blastocystis* sp. isolates obtained from cancer and HIV/AIDS patients, Parasitol. Res. 105 (2009) 1283–1286.
[45] S. Jantermtor, P. Pinlaor, K. Sawadpanich, S. Pinlaor, A. Sangka, C. Wilailuckana, W. Wongsena, H. Yoshikawa, Subtype identification of *Blastocystis* spp. isolated from patients in a major hospital in northeastern Thailand, Parasitol. Res. 112 (2013) 1781–1786.
[46] O. Sanpool, P. Laoraksawong, P. Janwan, P.M. Intapan, K. Sawanyawisuth, T. Thanchomnang, Y. Changtrakul, W. Maleewong, Genetic subtypes of *Blastocystis* isolated from Thai hospitalized patients in northeastern Thailand, Southeast Asian, J. Trop. Med. Public Health 46 (2015) 184.
[47] K.H.S. Wong, G.C. Ng, R.T.P. Lin, H. Yoshikawa, M.B. Taylor, K.S.W. Tan, Predominance of subtype 3 among *Blastocystis* isolates from a major hospital in Singapore, Parasitol. Res. 102 (2008) 663–670.
[48] L.D.N. Nguyen, N. Gantois, T.T. Hoang, B.T. Do, J. Desramaut, D. Naguib, T.N. Tran, A.D. Truong, G. Even, G. Certad, First epidemiological survey on the prevalence and subtypes distribution of the enteric parasite *Blastocystis* sp. in Vietnam, Microorganisms 11 (2023) 731.
[49] N.A. Mohammad, H.M. Al-Mekhlafi, T.S. Anuar, Genetic diversity of *Blastocystis* isolates from symptomatic and asymptomatic Orang Asli in Pahang, Malaysia, Southeast Asian, J. Trop. Med. Public Health 49 (2018) 189–197.
[50] K. Nithyamathi, S. Chandramathi, S. Kumar, Predominance of *Blastocystis* sp. infection among school children in Peninsular Malaysia, PLoS One 11 (2016) e0136709.
[51] A. Palasuwan, D. Palasuwan, A. Mahittikorn, R. Chiabchalard, V. Combes, S. Popruk, Subtype distribution of *Blastocystis* in communities along the Chao Phraya river, Thailand, Korean J. Parasitol. 54 (2016) 455.
[52] S. Popruk, R. Udonsom, K. Koompapong, A. Mahittikorn, T. Kusolsuk, J. Ruangsittichai, A. Palasuwan, Subtype distribution of *Blastocystis* in Thai-Myanmar border, Thailand, Korean J. Parasitol. 53 (2015) 13.
[53] R. Udonsom, R. Prasertbun, A. Mahittikorn, H. Mori, T. Changbunjong, C. Komalamisra, A. Pintong, Y. Sukthana, S. Popruk, *Blastocystis* infection and subtype distribution in humans, cattle, goats, and pigs in central and western Thailand, Infect. Genet. Evol. 65 (2018) 107–111.
[54] A. Yowang, A.D. Tsaousis, T. Chumphonsuk, N. Thongsin, N. Kullawong, S. Popluechai, E. Gentekaki, High diversity of *Blastocystis* subtypes isolated from asymptomatic adults living in Chiang Rai, Thailand, Infect. Genet. Evol. 65 (2018) 270–275.
[55] U. Thathaisong, S. Siripattanapipong, M. Mungthin, D. Pipatsatitpong, P. Tan-ariya, T. Naaglor, S. Leelayoova, Identification of *Blastocystis* subtype 1 variants in the home for girls, Bangkok, Thailand, Am. J. Trop. Med. Hyg. 88 (2013) 352.
[56] H. Yoshikawa, M. Tokoro, T. Nagamoto, S. Arayama, P.B.S. Asih, I.E. Rozi, D. Syafruddin, Molecular survey of *Blastocystis* sp. from humans and associated animals in an Indonesian community with poor hygiene, Parasitol. Int. 65 (2016) 780–784.
[57] O. Sanpool, S. Laymanivong, T. Thanchomnang, R. Rodpai, L. Sadaow, I. Phosuk, W. Maleewong, P.M. Intapan, Subtype identification of human *Blastocystis* spp. isolated from Lao People's Democratic Republic, Acta Trop. 168 (2017) 37–40.
[58] Y. Kesuma, A. Firmansyah, S. Bardosono, I.P. Sari, A. Kurniawan, *Blastocystis* ST-1 is associated with irritable bowel syndrome-diarrhoea (IBS-D) in Indonesian adolescences, Parasite Epidemiol, Control 6 (2019) e00112.
[59] A.M. Abdulsalam, I. Ithoi, H.M. Al-Mekhlafi, A.M. Al-Mekhlafi, A. Ahmed, J. Surin, Subtype distribution of *Blastocystis* isolates in Sebha, Libya, PLoS One 8 (2013) e84372.
[60] J. Forsell, M. Granlund, L. Samuelsson, S. Koskiniemi, H. Edebro, B. Evengård, High occurrence of *Blastocystis* sp. subtypes 1–3 and *Giardia intestinalis* assemblage B among patients in Zanzibar, Tanzania, Parasit. Vectors 9 (2016) 1–12.
[61] C.S. Poulsen, A.M. Efunshile, J.A. Nelson, C.R. Stensvold, Epidemiological aspects of *Blastocystis* colonization in children in Ilero, Nigeria, Am. J. Trop. Med. Hyg. 95 (2016) 175.

[62] V. Di Cristanziano, R. D' Alfonso, F. Berrilli, F.S. Sarfo, M. Santoro, L. Fabeni, E. Knops, E. Heger, R. Kaiser, A. Dompreh, Lower prevalence of *Blastocystis* sp. infections in HIV positive compared to HIV negative adults in Ghana, PLoS One 14 (2019) e0221968.
[63] A.F. Malheiros, C.R. Stensvold, C.G. Clark, G.B. Braga, J.J. Shaw, Molecular characterization of *Blastocystis* obtained from members of the indigenous Tapirapé ethnic group from the Brazilian Amazon region, Brazil, Am. J. Trop. Med. Hyg. 85 (2011) 1050.
[64] J.D. Ramírez, L.V. Sánchez, D.C. Bautista, A.F. Corredor, A.C. Flórez, C.R. Stensvold, *Blastocystis* subtypes detected in humans and animals from Colombia, Infect. Genet. Evol. 22 (2014) 223–228.
[65] W.D. Helenbrook, W.M. Shields, C.M. Whipps, Characterization of *Blastocystis* species infection in humans and mantled howler monkeys, *Alouatta palliata aequatorialis*, living in close proximity to one another, Parasitol. Res. 114 (2015) 2517–2525.
[66] A.P. Oliveira-Arbex, É.B. David, S. Guimarães, *Blastocystis* genetic diversity among children of low-income daycare center in Southeastern Brazil, Infect. Genet. Evol. 57 (2018) 59–63.
[67] R. Nagel, L. Cuttell, C.R. Stensvold, P.C. Mills, H. Bielefeldt-Ohmann, R.J. Traub, *Blastocystis* subtypes in symptomatic and asymptomatic family members and pets and response to therapy, Intern. Med. J. 42 (2012) 1187–1195.
[68] J.M. van Hattem, M.S. Arcilla, C. Schultsz, M.C. Bootsma, N. Verhaar, S.P. Rebers, A. Goorhuis, M.P. Grobusch, J. Penders, M.D. de Jong, Carriage of *Blastocystis* spp. in travellers - a prospective longitudinal study, Travel Med. Infect. Dis. 27 (2019) 87–91.
[69] A. Taghipour, E. Javanmard, H. Mirjalali, A. Haghighi, P. Tabarsi, M.R. Sohrabi, M.R. Zali, *Blastocystis* subtype 1 (allele 4): Predominant subtype among tuberculosis patients in Iran, Comp. Immunol. Microbiol. Infect. Dis. 65 (2019) 201–206.
[70] J.D. Ramírez, A. Sánchez, C. Hernández, C. Flórez, M.C. Bernal, J.C. Giraldo, P. Reyes, M.C. López, L. García, P.J. Cooper, Geographic distribution of human *Blastocystis* subtypes in South America, Infect. Genet. Evol. 41 (2016) 32–35.
[71] V. Jinatham, S. Maxamhud, S. Popluechai, A.D. Tsaousis, E. Gentekaki, *Blastocystis* One Health approach in a rural community of Northern Thailand: prevalence, subtypes and novel transmission routes, Front. Microbiol. 12 (2021) 746340.
[72] K. Vaisusuk, W. Saijuntha, S. Sedlak, T. Thanchomnang, W. Pilap, W. Suksavate, C.R. Stensvold, C. Tantrawatpan, *Blastocystis* subtypes detected in long-tailed macaques in Thailand—further evidence of cryptic host specificity, Acta Trop. 184 (2018) 78–82.
[73] A.A. Rauff-Adedotun, S.N. Mohd Zain, M.T. Farah Haziqah, Current status of *Blastocystis* sp. in animals from Southeast Asia: a review, Parasitol. Res. 119 (2020) 3559–3570.
[74] C. Tantrawatpan, K. Vaisusuk, T. Thanchomnang, W. Pilap, W. Sankamethawee, W. Suksavate, W. Chatan, N. Bunchom, O. Kaewkla, C.R. Stensvold, Distribution of *Blastocystis* subtypes isolated from various animal hosts in Thailand, Res. Vet. Sci. 162 (2023) 104939.
[75] J.S.Y. Hublin, J.G. Maloney, M. Santin, *Blastocystis* in domesticated and wild mammals and birds, Res. Vet. Sci. 135 (2021) 260–282.
[76] L.E. Gamalo, B. Sabanal, A. Ang, Three decades of Philippine nonhuman primate studies: research gaps and opportunities for Philippine primatology, Primates 62 (2021) 233–239.
[77] T.C. Tan, P.C. Tan, R. Sharma, S. Sugnaseelan, K.G. Suresh, Genetic diversity of caprine *Blastocystis* from Peninsular Malaysia, Parasitol. Res. 112 (2013) 85–89.
[78] A. McCain, L. Gruneck, S. Popluechai, A.D. Tsaousis, E. Gentekaki, Circulation and colonisation of *Blastocystis* subtypes in schoolchildren of various ethnicities in rural northern Thailand, Epidemiol. Infect. 151 (2023) e77.
[79] S. Khaled, N. Gantois, A.T. Ly, S. Senghor, G. Even, E. Dautel, R. Dejager, M. Sawant, M. Baydoun, S. Benamrouz-Vanneste, Prevalence and subtype distribution of *Blastocystis* sp. in Senegalese school children, Microorganisms 8 (2020) 1408.
[80] N. Gantois, A. Lamot, Y. Seesao, C. Creusy, L.-L. Li, S. Monchy, S. Benamrouz-Vanneste, J. Karpouzopoulos, J.-L. Bourgain, C. Rault, First report on the prevalence and subtype distribution of *Blastocystis* sp. in edible marine fish and marine mammals: a large scale-study conducted in Atlantic Northeast and on the coasts of Northern France, Microorganisms 8 (2020) 460.
[81] M. Farah Haziqah, N. Asyiqin, M. Mohd Khalid, K. Suresh, A. Rajamanikam, P. Chandrawathani, S. Mohd Zain, Current status of *Blastocystis* in cockroaches, Trop. Biomed. 34 (2017) 741–745.
[82] C. Valenca-Barbosa, T.C.B. do Bomfim, B.R. Teixeira, R. Gentile, S.F. da C. Neto, B.S.N. Magalhães, D. de A. Balthazar, F.A. da Silva, R. Biot, C.M. d'Avila Levy, Molecular epidemiology of *Blastocystis* isolated from animals in the state of Rio de Janeiro, Brazil, PLoS One 14 (2019) e0210740.
[83] L. Ma, Y. Zhang, H. Qiao, S. Li, H. Wang, N. Zhang, X. Zhang, Cockroach as a vector of *Blastocystis* sp. is risk for golden monkeys in Zoo, Korean J. Parasitol. 58 (2020) 583.
[84] S. Leelayoova, S. Siripattanapipong, U. Thathaisong, T. Naaglor, P. Taamasri, P. Piyaraj, M. Mungthin, Drinking water: a possible source of *Blastocystis* spp. subtype 1 infection in schoolchildren of a rural community in central Thailand, Am. J. Trop. Med. Hyg. 79 (2008) 401–406.
[85] I.L. Lee, T.C. Tan, P.C. Tan, D.R. Nanthiney, M.K. Biraj, K.M. Surendra, K.G. Suresh, Predominance of *Blastocystis* sp. subtype 4 in rural communities, Nepal, Parasitol. Res. 110 (2012) 1553–1562.
[86] V. Jinatham, C. Nonebudsri, T. Wandee, S. Popluechai, A.D. Tsaousis, E. Gentekaki, *Blastocystis* in tap water of a community in northern Thailand, Parasitol. Int. 91 (2022) 102624.
[87] V. Jinatham, T. Wandee, C. Nonebudsri, S. Popluechai, A.D. Tsaousis, E. Gentekaki, *Blastocystis* subtypes in raw vegetables from street markets in northern Thailand, Parasitol. Res. 122 (2023) 1027–1031.
[88] T.R. Riabi, H. Mirjalali, A. Haghighi, M.R. Nejad, M.A. Pourhoseingholi, P. Poirier, F. Delbac, I. Wawrzyniak, M.R. Zali, Genetic diversity analysis of *Blastocystis* subtypes from both symptomatic and asymptomatic subjects using a barcoding region from the 18S rRNA gene, Infect. Genet. Evol. 61 (2018) 119–126.
[89] X. Villamizar, A. Higuera, G. Herrera, L.R. Vasquez-A, L. Buitron, L.M. Muñoz, F.E. Gonzalez-C, M.C. Lopez, J.C. Giraldo, J.D. Ramírez, Molecular and descriptive epidemiology of intestinal protozoan parasites of children and their pets in Cauca, Colombia: a cross-sectional study, BMC Infect. Dis. 19 (2019) 1–11.
[90] S. Mattiucci, B. Crisafi, S. Gabrielli, M. Paoletti, G. Cancrini, Molecular epidemiology and genetic diversity of *Blastocystis* infection in humans in Italy, Epidemiol. Infect. 144 (2016) 635–646.
[91] J.D. Ramírez, C. Flórez, M. Olivera, M.C. Bernal, J.C. Giraldo, *Blastocystis* subtyping and its association with intestinal parasites in children from different geographical regions of Colombia, PLoS One 12 (2017) e0172586.
[92] S. Gabrielli, F. Furzi, E. Brianti, G. Gaglio, E. Napoli, L. Rinaldi, R.A. Alburqueque, M. Paoletti, S. Mattiucci, Molecular detection of *Blastocystis* from animals in Italy: subtypes distribution and implications for the zoonotic transmission, Res. Sq. (2020), https://doi.org/10.21203/rs.3.rs-34122/v1.
[93] Y. Yan, S. Su, J. Ye, X. Lai, R. Lai, H. Liao, G. Chen, R. Zhang, Z. Hou, X. Luo, *Blastocystis* sp. subtype 5: a possibly zoonotic genotype, Parasitol. Res. 101 (2007) 1527–1532, https://doi.org/10.1007/s00436-007-0672-y.