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Blastocystis genetic diversity in animal and human samples from different departments of Colombia using complete sequencing of the 18S rRNA gene (SSU rRNA) by Oxford Nanopore Technologies (ONT)

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Abstract

Blastocystis is an intestinal microeukaryote that has raised attention due to its wide distribution in animals and humans. The risk of zoonotic circulation primarily arises from close contact with infected animals. Therefore, the following study aimed to evaluate the diversity and frequency of *Blastocystis* subtypes in Colombian human and animal samples using complete sequencing of the 18S rRNA gene. For this purpose, 341 human stool samples and 277 animal fecal samples (from cattle, sheep, goat, pigs, cats, and dogs), were collected from different Colombian regions and analyzed using PCR-based detection and full-length 18S SSU rRNA gene Next-Generation Sequencing (NGS). Among the 618 samples from both hosts, humans and animals, the results revealed widespread *Blastocystis* frequency, with 48.09% (n=164) in humans and 31.4% (n=87) detection in animals. Dogs, cats, sheep, pigs, and wild animals tested positive, aligning with global prevalence patterns. Also, 29 human samples and 23 animal samples were sequenced using ONT technology from which 11 long-read unique sequences were generated and cluster with their compared reference sequences. The subtype distribution varied within hosts, detecting ST1 and ST3 in both human and animal samples. Subtypes ST5, ST10, ST14, ST15, ST21, ST24, ST25 and ST26 were limited to animals hosts, some of which are considered to have zoonotic potential. On the other hand, ST2 was found exclusively in human samples from Bolivar region. Mixed infections occurred in both animal and humans, 60.86% and 27.58% respectively.

Moreover, to our knowledge, this is the first study in Colombia identifying ST15 in pigs and ST25 in sheep. The subtypes (STs) identified in this study indicate that certain animals may serve as reservoirs with the potential for zoonotic transmission. The identification of zoonotic subtypes highlights the use of Next Generation Sequencing as the depth and resolution of the sequences increases providing insights into STs of medical and veterinarian significance. It also reveals the coexistence of diverse subtypes among hosts. Further research is essential for understanding transmission dynamics, health implications, and detection strategies for *Blastocystis* occurrence in animals and humans, mainly associated to the role of animals as reservoirs and their close interaction with humans.

Key words: *Blastocystis*, Subtypes, STs, Colombia, Animals, Humans, SSU rRNA, Sequencing

1. Introduction

Blastocystis, an enteric protist classified within the chromista kingdom and recognized as a stramenopile (Stensvold et al., 2020), predominantly colonizes the gastrointestinal tract of diverse hosts (Qi et al., 2020; Yoshikawa et al., 2016). It exhibits a widespread global distribution, with prevalence estimates ranging from 0.5% to 24% in developed nations and escalating to 50-100% in developing countries (Alfellani et al., 2013a). The primary transmission route involves the fecal-oral pathway (Lee et al., 2012; Li et al., 2007), facilitated by water, food, and contact with certain animals as the most probable propagation mechanisms (Londoño-Franco et al., 2014; Maloney and Santin, 2021).

While the manifestation of symptoms due to *Blastocystis* infections in animals remains unclear, a substantial prevalence of subtypes has been documented across various species. Noteworthy, some reports provide evidence for *Blastocystis* potential zoonotic transmission between humans and animals (Hublin et al., 2020; Ramírez et al., 2017). In contrast, among human populations, *Blastocystis* has been identified as a possible cause of gastrointestinal and cutaneous symptoms. Interestingly, its frequent identification in asymptomatic patients adds complexity to understanding the intricate relationship between infection and disease (Ajjampur and Tan, 2016; Casero et al., 2015; Clark et al., 2013; Wawrzyniak et al., 2013). Additionally, there are ongoing investigations into whether it acts as a pathogen or as a part of the healthy intestinal microbiota (Deng et al., 2021).

This microeukaryote exhibits a wide genetic diversity (C. Rune Stensvold et al., 2012; Stensvold et al., 2020), currently 40 subtypes (ST) have been reported, based on nucleotide polymorphism within the small subunit of ribosomal RNA (rRNA-18S)

(Hernández-Castro et al., 2023; Higuera et al., 2021; Rondón et al., 2023; Santin et al., n.d.; Stensvold, 2013; Stensvold et al., 2023; Stensvold and Clark, 2020). Certain subtypes display moderate specificity towards human and animal hosts, while others are exclusively identified in animals (Alfellani et al., 2013a; Barbosa et al., 2017; Ramirez et al., 2016). ST1 to ST4, are predominantly detected from humans (90%). Meanwhile, ST5 to ST8 are primarily associated with animal hosts, such as sheep, pigs, dogs, cats, rodents, and birds. Remarkably, ST5 has also been detected in amphibians and was recently reported in human samples within a Colombian population by Vega et al. (Alfellani et al., 2013b; Vega Romero, 2020). Conversely, ST9 has exclusively reported in human hosts (Stensvold and Clark, 2016). ST10 to ST17 predominantly inhabit non-human hosts, including marsupials, goats, non-human primates, elephants, wild birds, and sheep (Maloney et al., 2018, 2021a; Roberts et al., 2013). Although subtypes ST12, ST13, and ST16, are commonly associated with non-human hosts, researchers such as Ramírez et al. and Osorio-Pulgarín et al have detected them in human samples (Jiménez et al., 2019; Osorio-Pulgarin et al., 2021; Ramirez et al., 2016). ST21 to ST26 have been documented in cattle and sheep, ST21 was identified in antelopes (*Kobus ellipsiprymnus*) (Maloney et al., 2021a; Zhao et al., 2017) and ST24 in Llamas (*Lama glama*) (Higuera et al., 2021). ST27 and ST28 were documented in samples from wild birds (Maloney et al., 2020a), while ST29 was detected in chickens (Maloney et al., 2021a). Recent additions include ST30 and ST31, characterized in white-tailed deer (*Odocoileus virginianus*) (Maloney et al., 2021b). Additionally, Higuera et al. reported subtype ST32 in Colombian cattle and goats (Higuera et al., 2021), whereas ST33 and ST34 were found in Colombian horses (Baek et al., 2022). Novel ST41 identified in a Colombian patient undergoing a colorectal cancer screening (Hernández-Castro et al., 2023). Furthermore, genetic diversity varies considerably among subtypes and even within individual subtypes, reflecting pronounced genetic heterogeneity which could be associated with biological features and phenotypic effect on the host (Stensvold, 2013).

Molecular characterization of *Blastocystis* involves techniques such as PCR-based partial amplification of the rRNA-18S gene (600 pb), followed by Sanger sequencing, which leads to subtype assignment via database comparison. Nonetheless, this method is known for missing mixed infections by omitting subtypes in low abundance, leading to underestimated results in terms of subtype diversity. Conversely, Illumina sequencing has been adopted for *Blastocystis* subtyping, demonstrating heightened sensitivity and specificity, especially in scenarios involving mixed infections, low abundance. This technique notably provides valuable information of those subtypes with zoonotic potential such as ST1 to ST5 (Maloney et al., 2019).

Emerging as a fundamental tool, third-generation sequencing methods, such as Oxford Nanopore Technologies (ONT) using the MinION device, have been used to obtain complete sequences of the *Blastocystis* 18S SSU rRNA gene (1800 pb), increasing the accuracy of genetic information obtained, enhancing confidence in the identified subtypes. The MinION's unique attributes, like generating long reads of up to 882 kb and avoiding the use of multiple primers or repeated PCRs, enabling the identification of novel subtypes and proper phylogenetic analysis (Jain et al., 2018; Maloney and Santin, 2021). Furthermore, MinION reduces sequencing duration from days to hours, diminishes PCR-induced biases, and continuous improvements sequence processing and software lead to accurate individual reads up to 99% (Bowden et al., 2019; Hernández et al., 2023; Katoh and Standley, 2013; Maloney et al., 2020b).

The generation of full-length 18S SSU rRNA gene sequences not only offers reference material for subtype identification and robust phylogenetic lineage development but also proves valuable for designating new subtypes. The complete gene sequence meets the 4% minimum divergence threshold, preventing invalid subtype creation or nomenclatural errors (Higuera et al., 2021; Stensvold, 2013; Stensvold and Clark, 2020). Indeed, during the last years with this technology it was possible to identify eight new subtypes, such as ST32 in Colombian goats and cows, ST33 and ST34 in Colombian horses (Baek et al., 2022), among others up to ST38 (Hernández et al., 2023; Maloney et al., 2023). This methodology facilitates a deeper understanding of mixed infections within single samples, reducing the necessity of obtaining individual reference sequences via pure cultures for each subtype. Its utility improve the understanding of transmission dynamics, pathogenicity, and host specificity in a genetically diverse microorganism like *Blastocystis* (Maloney et al., 2020b).

In Colombia, few studies have focused on the molecular characterization of *Blastocystis* within animal and human populations using ONT. While the National Survey of Intestinal Parasitology suggests a prevalence range of 3.6% to 60.3% (Ministerio de Salud y Protección Social, 2015), distinct distributions of subtypes have been identified across departments such as Guainía, Quindío, Tolima, Cundinamarca, Boyacá, Amazonas, Cauca, Antioquia, Bolívar, Córdoba and Casanare (Castañeda et al., 2020; Higuera et al., 2020a; Jiménez et al., 2019; Ramirez et al., 2016; Vega Romero, 2020; Villamizar et al., 2019). Predominantly, Andean, and Caribbean regions exhibit high diversity and prevalence of intestinal parasites, including *Blastocystis* (Higuera et al., 2020b). However, research has primarily focused on intestinal parasitism linked to nutritional deficiencies and infantile polyparasitism (Osorio-Pulgarin et al., 2021; Ramírez et al., 2017).

The molecular characterization of intestinal protists such as *Blastocystis* is essential for understanding its epidemiology, and frequency, comprehending transmission dynamics and host specificity. Due to its high frequency, *Blastocystis* play a significant role in public health and disease research implications. Despite the elevated *Blastocystis* prevalence in Colombia, most studies have employed PCR and sequencing techniques (Sanger or Illumina) to analyze a fragment of the SSU rRNA gene, resulting in an increasing focus on animal hosts and a decreasing emphasis on human subtyping. However, comprehensive gene sequences remain sparse, with limited studies exploring both host types. Oxford Nanopore's MinION technology has bridged this gap in animal hosts, even though with limited human research or combined host analyses. Consequently, complete rRNA-18S gene sequences of diverse *Blastocystis* subtypes in Colombia remain unavailable. Therefore, this study seeks to address this gap by characterizing the genetic and epidemiological diversity of *Blastocystis* in both animal and human hosts across diverse Colombian regions, thereby generating full-length rRNA-18S gene sequences.

2. Materials and Methods

2.1 Study Population and Sample Collection

The human samples were originally collected in a previous study during which the identification of *Blastocystis* was also carried out, but were utilized in the current study. A total of 341 stool samples were collected from individuals ranging various age groups from 1 to 70 years old, (mean: 5 years - standard deviation: 6 years) residing in both rural and urban settings. Convenience sampling was conducted across diverse regions of Colombia, including departments such as Casanare (n=53), Cauca (n=258), and Bolívar (n=30) (Higuera et al., 2020b).

Additionally, fecal samples were included from 277 apparently healthy domestic animals and livestock representing distinct species. These samples were collected from rural areas in Boyacá (Jenesano, Gámeza and Paipa) and urban areas in Bogotá, including Pigs *(Sus scrofa)* (n=36), Cattle *(Bos taurus)* (n=29), Sheep *(Ovis aries*) (n=38), Goats (*Capra aegagrus hircus*) (n=20), Cats (*Felis catus*) (n=96) Dogs (*Canis lupus familiaris*) (n= 35), this animals were apparently healthy and have no exhibit digestive symptoms. Sampling was conducted during the period from November to March 2022, large animal (cows, pigs, sheep, and goats) sampling was performed by anal conduct collection, around 300 mg of stool were collected in transparent sterile container for coprological analysis. In the case of small animals such as cats and dogs with minimal contact between the animal and the keepers, around 200 mg of stool was collected from the ground using transparent sterile container for coprological analysis.

Also, stool samples were collected from zoo animals (n=23) whose species are shown in Supplementary Table 2. These samples were collected in November 2019 from wild mammals rescued from illegal trafficking and held in captivity in Cubará, Boyacá. Despite their previous exposure to humans and other animals, these animals exhibited no signs of intestinal infection. They were housed in dedicated enclosures and provided with a specific diet under veterinary supervision and had access to cistern water sourced from a stream-connected reservoir. No deworming protocol had been initiated previously. Stool samples were collected both individually and in groups (from animals of the same species housed together detailed in Supplementary Table 2), with minimal contact between the animals and keepers, approximately 200 mg of stool were collected from the ground using Eppendorf tubes containing ethanol (Cruz-Saavedra et al., 2023).

The specific geographical origins of these samples are displayed in Figure 1. All samples were stored at -30°C until DNA extraction without any preservative intervention. Samples that were unavailable during molecular analysis were excluded, and due to the absence of accompanying clinical information, clinical parameters were not established as exclusion criteria.

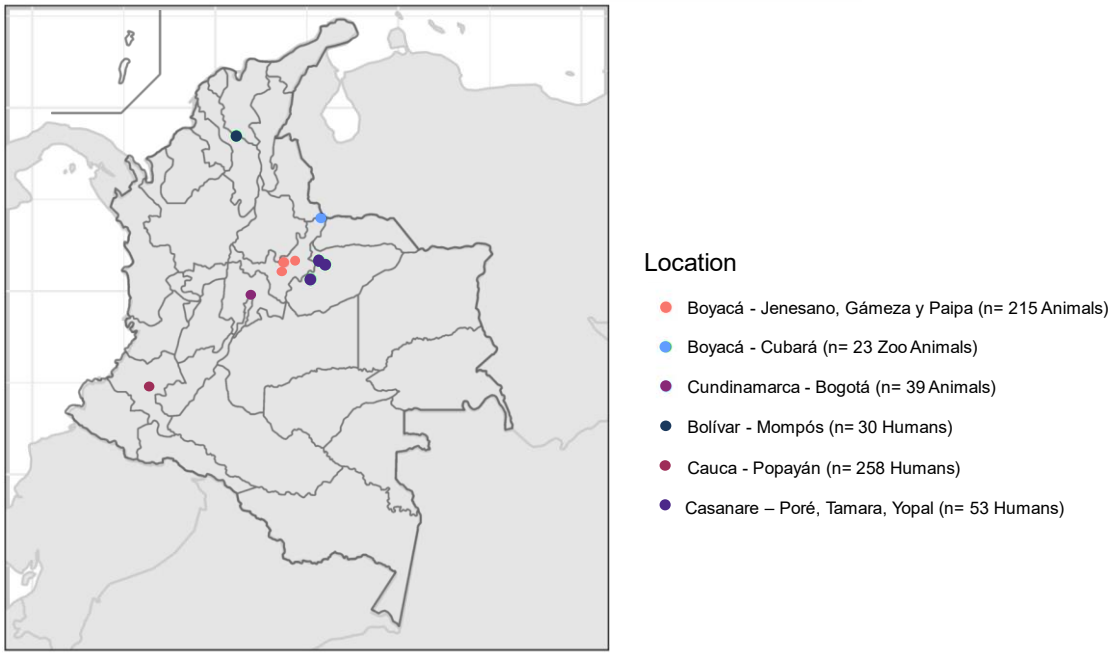

Figure 1. Geographic Distribution of Sample Collection. Exact geographical location from the municipalities where the samples were collected are indicated in dots of distinct colors by department. The total number of samples and host from which those were taken are exhibited in the Location legend.

2.2 DNA extraction and *Blastocystis* detection via PCR

DNA extraction and *Blastocystis* detection had been previously performed in human samples by Higuera *et al*., resulting in the identification of 164 positive samples (Higuera et al., 2020b). Subsequently, a survey was conducted to assess the current availability of these samples, revealing that 111 of them were still available (Supplementary Table 1). For animal samples, prior to DNA extraction, 300 µL of the stool were washed with sterile saline and phosphate buffer (PBS 1X) three times. Subsequently, 600 mL of PBS was added to each sample by vortexing for 30 seconds and centrifugation at 3000 rpm for one minute. The supernatant was discarded, and DNA was extracted from the fecal pellet using the Norgen Stool DNA Isolation Kit, Norgen Biotek Corp (Thorold, ON, Canada), following the manufacturer's recommendations.

Blastocystis detection in animal samples followed the same methodology as previously described for human samples (Higuera et al., 2020b). The amplification of the rRNA-18S gene fragment was achieved by PCR, with a final volume of which contained 0.28X (3.5 µL) GoTaq Green Master Mix (Promega®, Madison, Wisconsin, USA), 2 µL of template DNA, and 1 uM of each primer with a final volume of 9 µL. The primers used were Blasto FWD F5 (5′-GGTCCGGTGAACACTTTGGATTT-3′) and Blasto R F2 (5′-CCTACGGAAACCTTGTTACGACTTCA-3′) (Christen Rune Stensvold et al., 2012). Thermal cycling starts with 95°C for 5 min, then 35 cycles of 95°C for 15 s, 58°C for 1 min, and 72°C for 30 s; 72°C for 10 min. The size of each amplicon was evaluated by 2% agarose gel electrophoresis stained with SYBR™ Safe (Invitrogen™, Carlsbad, CA, USA (Stensvold and Clark, 2016; Zhao et al., 2017). Human samples did not undergo this procedure, as *Blastocystis* identification had been previously performed using this method in the study by Higuera and colleagues (Higuera et al., 2020b).

2.3 PCR Amplification and Sequencing of the Full-Length SSU rRNA 18S Gene by Oxford Nanopore MinION

Molecular characterization of *Blastocystis* was performed on PCR-positive samples from both human and animal hosts. DNA from these samples underwent PCR to generate amplicons of the complete rRNA-18S gene, approximately 1,800 bp in length, using 1 µM of primers (Af) SSU-F1 (5-AAC CTG GTT GAT CCT GCC AGT AGT AGT C-3) and (Br) SSU-R1 (5-TGA TCC TTC TGC AGG TTC ACC TAC G-3) forward and reverse, respectively. This was carried out utilizing 0.3375 U (12.5 µL) high-fidelity proofreading polymerase contained in KAPA HiFi Hot Start Ready-mix (KAPA Biosystems, Cape Town, South Africa) with a reaction volume of 25 µL. The thermal cycling involved initial denaturation at 98°C for 5 minutes, followed by 35 amplification cycles of 20 seconds at

98°C, 45 seconds at 60°C, and 90 seconds at 72°C. A final extension of 5 minutes concluded the process (Maloney et al., 2020b).

The Oxford Nanopore MinION platform was utilized for characterization, following the protocol established by Maloney *et al*. in 2020 (Maloney et al., 2021b, 2020b). PCR amplicons were purified using 0.5 × AMPure XP beads (Beckman Coulter Brea, CA, USA) (Higuera et al., 2021; Maloney et al., 2021b). The SQK-LSK109 and SQK-LSK110 ligation sequencing kits from Oxford Nanopore Technologies (ONT) were employed for library preparation, following the manufacturer's instructions for PCR coding amplicons (PBAC12_9112_v110_revB_10Nov2020). The EXP-PBC001 PCR Barcoding Kit from ONT, Oxford, UK, was used in conjunction with ligation kits for barcoding each sample. Subsequent purification steps ensured the loading of 50 or 75 fmol into the flow cell R9 version. MinKNOW v20.10.06 software facilitated the sequencing runs (Maloney et al., 2020b).

2.4 Bioinformatics Analysis

Basecalling was performed using Guppy v4. Reads were visualized using UGENE v46.0 software. FASTQ reads were filtered to retain lengths between 1600 and 2100 bp nucleotides. Adapters utilized for full-length sequencing PCR were trimmed, and only reads with full-length direct and reverse adapters were retained. From the generated FASTA files, subtyping was accomplished through alignment based on the best match with full-length *Blastocystis* reference nucleotide sequences, obtained from the reference database (http://entamoeba.lshtm.ac.uk/blastorefseqs.htm, accessed on 03/02/2021) along with other accepted full-length ST sequences present in GenBank, utilizing CENTRIFUGE algorithm with the following parameters: -k 1 --min-totallen 800 --qc-filter. To validate the assigned STs, the BLAST algorithm was utilized (10 std slen" -perc_identity 95 -evalue 0.01 -max_target_seqs 1). The acquired full-length sequences were deposited in the GenBank database with the following accession numbers SAMN37309017 - SAMN37309027.

2.5 Phylogenetic Reconstruction

Phylogenetic reconstruction employed full-length reference sequences which include all currently published subtypes and full-length SSU rRNA sequences obtained in this study. Alignment was performed using the MAFFT v.7 algorithm, and the tree was rooted with *Proteromonas lacertae*, a closely related Stramenopile, as an outgroup (Maloney et al., 2020b). The maximum likelihood method was applied for phylogenetic reconstruction, under nucleotide substitution model: TIM3+F+R6. Support for clades was determined through 1000 bootstrapping replicates, genetic distances were calculated with Kimura 2-

parameter model using IQtree (Minh et al., 2020). The Interactive Tree of Live v32 tool facilitated tree editing and visualization (Letunic and Bork, 2016). Finally, phylogenetic network was also built in SplitsTree4 (Huson and Bryant, 2006) using the Neighbor-Net method.

2.6 Frequencies and Analysis of the Genetic Diversity *Blastocystis* Subtypes

Once STs were obtained and validated for the samples herein used, using ArcGIS software, a map was generated to visualize distribution of subtype frequencies in human and animal samples. This was represented through pie charts, aligned with specific geographical locations of each department or municipality included in the study. The geographic data was extracted from a compiled database of acquired sequences. Additionally, we constructed bar plots to illustrate mixed infection variations within each sample, considering hosts and geographical regions. To differentiate the identified subtypes, a distinct color pattern was implemented. Additionally, we utilized the alignment of the 11 concatenated sequences obtained for each *Blastocystis* subtype to calculate indices of diversity, polymorphic sites, the number of haplotypes, haplotype diversity, and conducted population structure tests such as Tajima's D, Fu and Li's D and F.

3. Results

3.1 *Blastocystis* Identification

A total of 277 animal feces samples were included in this study. Out of these, 87 samples (31.4%) tested positive for *Blastocystis* through PCR in both geographic regions—Bogotá and Boyacá. Specifically, within the Boyacá region, 76 samples (27.43%) from animal hosts were positive, which correspond as follows 62.8% (n=22) from dogs, 36.8% (n=21) from cats, 28.9% (n=11) from sheep, 52.7% (n=19) from pigs, and 13.6% (n=3) from zoo animals (*Odocoileus virginianus, Dasyprocta fuliginosa, and Ateles hybridus).* Additionally, 28.2% (n=11) of positive samples were collected from cats in Bogotá (Table 2). This suggests a wide detection of *Blastocystis* in both geographical areas. After subjecting the 87 samples to a second PCR for amplification of the full-length 18S SSU rRNA gene, positive results were obtained for 23 samples, corresponding to pigs (n=15), sheep (n=7) and zoo animals (n=1) belonging to *Ateles hybridus.* The presence of a distinct band in the electrophoresis gel confirmed these positive results.

A total of 111 human samples were considered, selected based on the detection of *Blastocystis* via Sanger sequencing of a 600 bp region of the SSU rRNA gene, as previously conducted by Higuera *et al* (Higuera et al., 2020b). These samples exhibited a high frequency of the protist in the Caribbean region, followed by the Orinoco. Out of

this group, 29 samples available (Bolívar n=10 and Casanare n=19) were subjected to Oxford Nanopore sequencing to obtain full-length sequences.

3.2 Phylogenetic Analysis

To verify the accuracy of the identified subtypes from the obtained full-length sequences of the SSU rRNA 18S gene, a phylogenetic reconstruction was conducted following the recommended guidelines (Stensvold and Clark, 2020). The eleven full-length sequences representing various subtypes (ST1, ST2, ST3, ST5, ST10, ST14, ST15, ST21, ST24, ST25, ST26) from different samples were included in this analysis. Employing the maximum likelihood method, the phylogenetic relationship among these sequences was determined. Notably, all the sequences clustered alongside the corresponding reference full-length sequences, further affirming their validity (Figure 2).

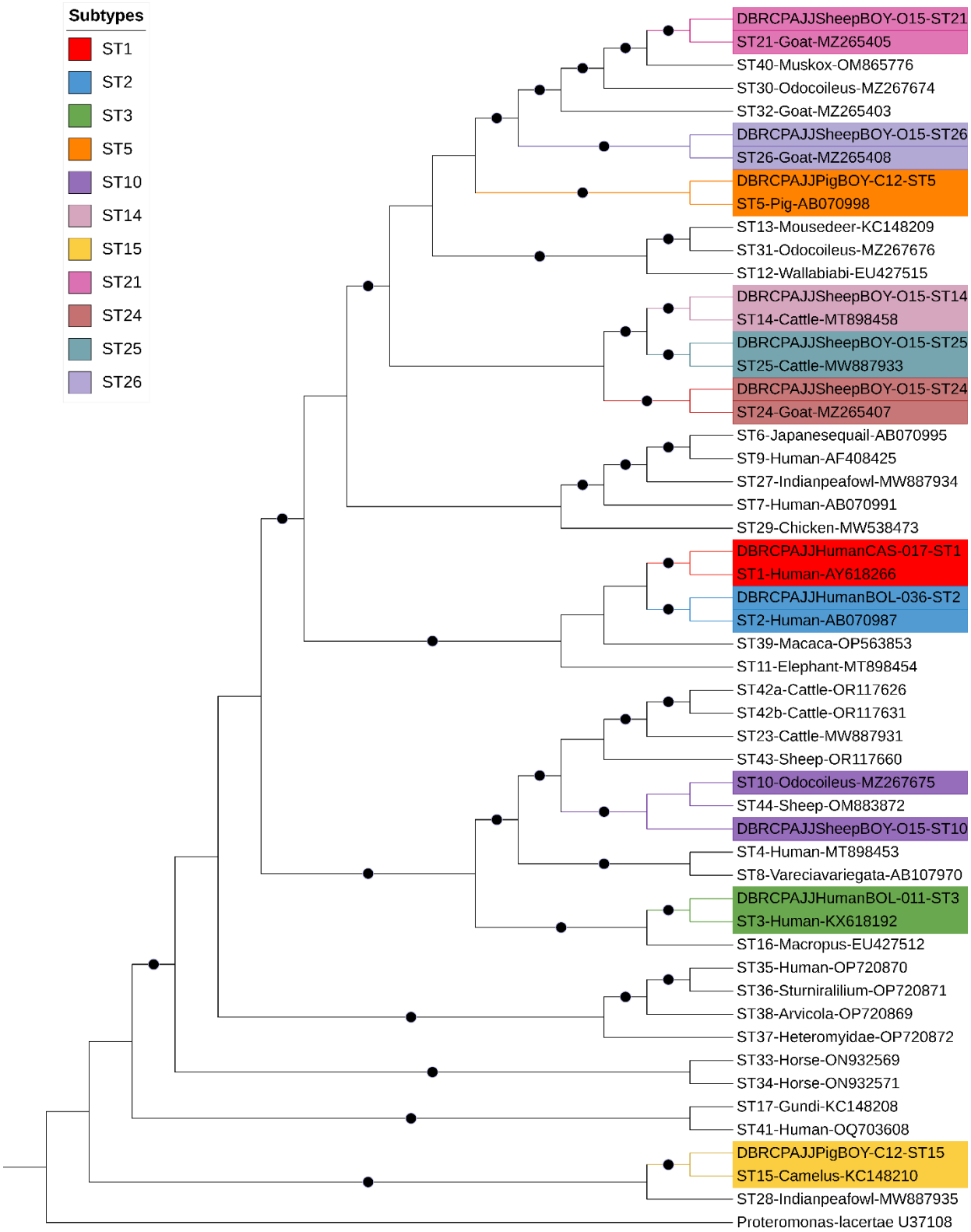

Figure 2. Phylogenetic Relationships from the full-length SSU rRNA gene sequences obtained in the present study, *Blastocystis* representative reference sequences from each accepted ST, and *Proteromonas lacertae* as the outgroup microorganism for rooting the tree.

3.3 *Blastocystis* Subtypes and Intra-Subtype Variation

Of the 198 PCR-positive samples from both human and animal hosts, 52 were sequenced for *Blastocystis* presence, 29 belonging to human samples and 23 to animal samples as follows: pigs (n= 15), sheep (n= 7) and zoo animals (n=1), yielding a total of 2,670,529,445 reads. After quality control by trimming and filtering, 158,732 reads remained for further analysis and phylogenetic reconstruction. Among the 52 sequenced samples, 11 distinct *Blastocystis* subtypes were identified throughout animal and human hosts, with the exception of sample 34, which had no assigned subtypes. The most prevalent subtype was ST1 detected in 43.13% (n=22) in both animal and human samples. Following closely was ST3, present in 39.21% (n=20) of samples from both hosts. Other subtypes were limited to either animal or human samples. Notably, ST2 was found only in human samples (7.84%, n=4), while subtypes ST5, ST15, ST10, ST24, ST25, and ST26 were found exclusively in animal samples, comprising 21.56% (n=11), 15.68% (n=8), 11.76% (n=6), 11.76% (n=6), 11.76% (n=6), and 9.80% (n=5) of the samples, respectively. ST14 and ST21 were the least frequent subtypes detected in animal samples (7.84%, n=4). The total of subtype frequencies exceeds 100% given detection of mixed infections with more than one *Blastocystis* subtype co-occurring within individual samples.

Genetic diversity indices were calculated by hosts (Human, Sheep and Pig), along with the entire dataset of STs (Total), showing the number of haplotypes equaled the number of sequences analyzed, indicating that each identified subtype was represented by unique sequences. The number of segregating sites in STs detected in human and sheep samples were greater than in pig samples. This finding was confirmed by analyzing the paired comparison between haplotypes and supported by the tree topology obtained from the phylogenetic network constructed in SplitsTree5, in which every subtype had its own branch (Supplementary Figure 1).

The analysis of genetic diversity and population structure in three distinct groups showed that pig samples have displayed the most pronounced genetic diversity, characterized by a π value of 0.14502, indicating a high degree of genetic variability compared to the other populations. human STs exhibits a moderate level of genetic diversity, as reflected by a π value of 0.14011. In contrast, sheep samples demonstrate a lower genetic diversity with a π value of 0.09301. In terms of population genetic structure pig subtypes had the highest R2 value of 0.5 compared with the other hosts which displays a comparatively lower degree of R2 values. In general, the populations evaluated had different levels of genetic diversity and population genetic structure (Supplementary Table 3).

3.4 *Blastocystis* Subtypes in Animal Hosts

A total of 23 animal samples were analyzed by Nanopore sequencing, As previously mentioned, sample 34, originating from a pig, did not yield a successful assignment using the employed methods. Hence, 22 samples were further analyzed. Across these animal samples, sequences from 10 distinct subtypes were obtained. Remarkably, ST15, which had not been previously reported in this country, was the second most common subtype, detected in 8 out of 22 samples (36.36%). ST5 was the most prevalent subtype, found in 11 out of 22 samples (50%). In terms of lower subtype frequencies, ST10, ST24, and ST25 were detected in 6 samples (27.27%). ST26 was identified in 5 out of 22 samples (22.72%), while ST1, ST14, and ST21 were each observed in 4 out of 22 samples (18.18%). Finally, ST3 was present in 3 out of 22 samples (13.63%) (Figure 3a). These results showed that in several samples were detected more than one subtype.

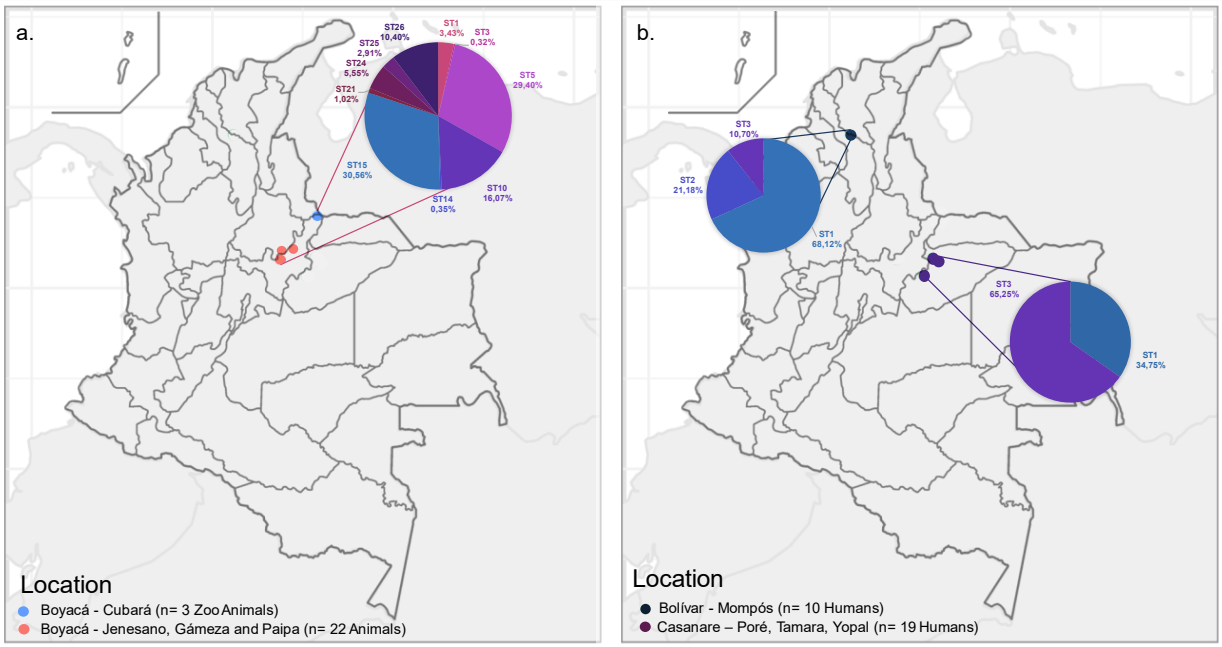

Figure 3. Geographical Distribution of *Blastocystis* Subtypes in Colombia. a. Animal Samples. b. Human samples. Marked with different colored dots, the precise locations where the sequenced samples were taken are shown on the map of Colombia. Pie charts with the percentages of subtypes by department are included. The host and number of samples used for this study are specified in the legend.

Sheep samples displayed a higher STs diversity in *Blastocystis* subtypes, with six subtypes identified: ST10, ST14, ST21, ST24, ST25, and ST26. Multiple subtypes were detected within individual samples from the same sheep host, indicating mixed infections. Most sheep samples 6/7 (85.71%) displayed mixed infections, with six out of seven samples testing positive for ST10 (85.71%) and five out of seven samples

(71.42%) testing positive for ST24, ST25, and ST26. Other subtypes were detected at lower frequencies: ST21 in three out of seven samples (42.85%) and ST14 in two out of seven samples (28.57%) (Figure 4a).

In contrast, pig samples exhibited lower diversity of STs, with four identified *Blastocystis* subtypes: ST1, ST3, ST5, and ST15. Most pig samples (64.28%) showed mixed infections, with 9 out of 14 samples displaying multiple subtypes. ST15 was the most prevalent subtype in pig samples, found in 78.57% (11 out of 14) of the samples, followed by ST15 in 57.14% (8 out of 14) of the samples. Other subtypes were detected at lower frequencies: ST3 in 28.57% of the samples (4 out of 14), and ST1 in 21.42% of the samples (3 out of 14). Additionally, only one subtype, ST1, was identified in the zoo animal *Ateles hybridus* (Figure 4a).

3.5 Variation of *Blastocystis* Subtypes in Human Hosts

The examination of human samples revealed a limited diversity in *Blastocystis* subtypes, detecting three previously reported STs: ST1, ST2, and ST3. The most prevalent subtype among the samples was ST1, constituting 62.06% of cases, followed by ST3 (51.72%), and ST2 (13.79%) (Figure 3b). These findings align with the assignments made by Higuera et al. using Sanger sequencing.

Upon analyzing the subtype diversity of human samples across different departments, Casanare exhibited the presence of two subtypes (ST1 and ST3), indicating comparatively lower STs diversity than Bolívar, where three STs were detected (ST1, ST2, and ST3). Intriguingly, the frequencies of the most abundant subtypes in these areas appeared to differ based on geographic location. In Casanare, the two identified STs were equally prevalent, representing 68.42% of the samples (n=13). In contrast, in Bolívar, ST1 emerged as the dominant subtype, observed in 50% of the samples (n=5), followed by ST2 in 40% of the samples (n=4), and ST3 in 30% of the samples (n=3) (Figure 2). Additionally, mixed infections were observed within this group of isolates. Among the 29 analyzed human samples, eight (27.58%) displayed co-infections with two subtypes. The combination of ST1 and ST3 was exclusively detected in Casanare (36.84%), while a mixed infection of ST1 and ST2 was identified in Bolívar (10%). The distribution of *Blastocystis* subtypes between Casanare and Bolívar demonstrated certain variations in occurrences of unique and multiple STs (Figure 4b).

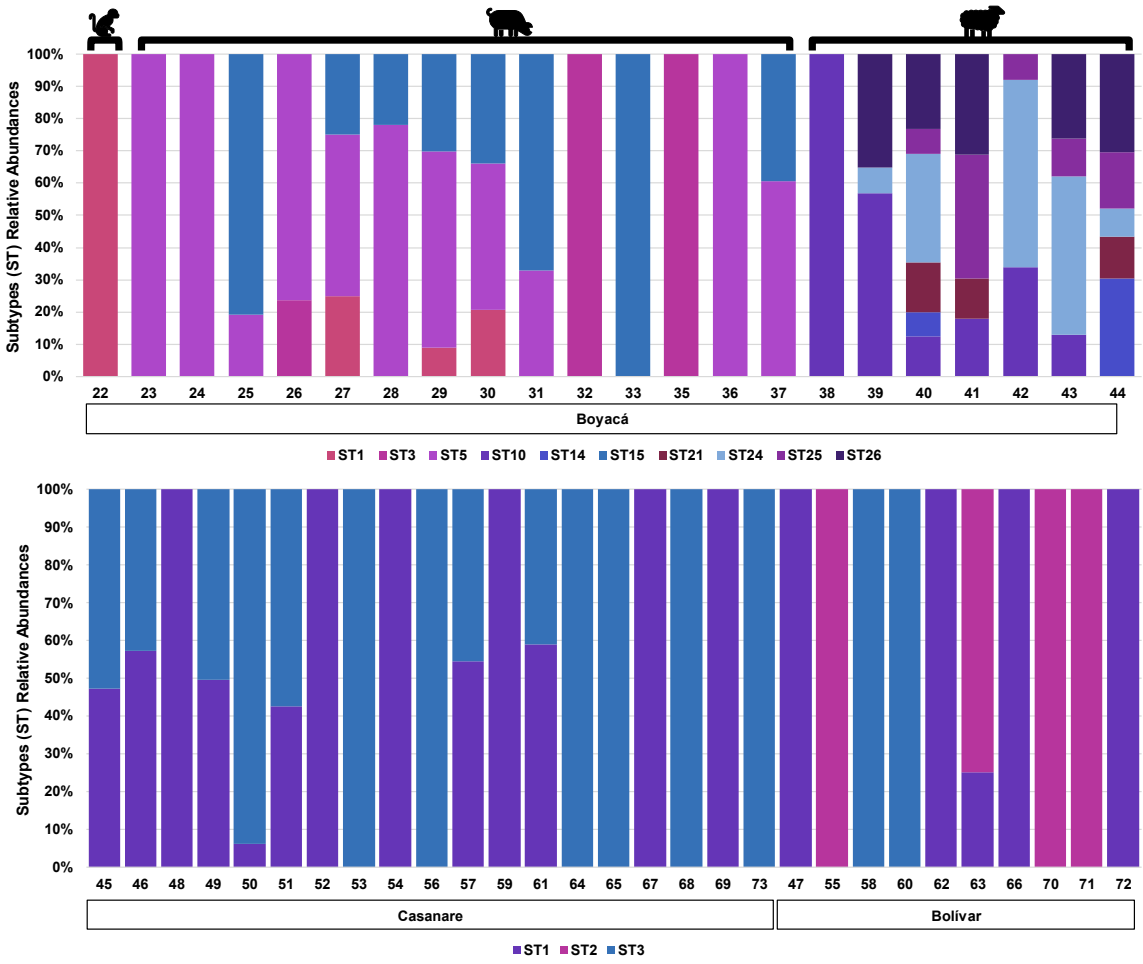

Figure 4. *Blastocystis* Variation between ST in animal and human hosts. In this study was performed the detection of mixed and unique infection from different *Blastocystis* subtypes. Bar plots were constructed by host to reveal the percentage of each ST detected by sample. All subtypes were assigned a color to distinguish them from each other. a. *Blastocystis* STs diversity in animal hosts. b. *Blastocystis* STs diversity in humans by department.

4. Discussion

The present study reports the frequency and distribution of *Blastocystis* subtypes in both animal and human hosts across various locations in Colombia, including Bogotá and Boyacá for animals, and Bolivar and Casanare for humans. Interestingly, *Blastocystis* was detected in all sampled locations, indicating a widespread distribution of the microeukaryote in these areas. The overall detection rate of *Blastocystis* in animal feces samples was 31.4%, with positive cases found in various animal hosts, including dogs, cats, sheep, pigs, and zoo animals. These findings reveal a lower frequency compared to previous reports in Colombia, where frequencies ranged between 67%-81.4% (Higuera et al., 2021; Onder et al., 2021). However, the observed prevalence is

consistent with reports from other countries in Europe and Asia, ranging from 8.9% to 43.6% (AbuOdeh et al., 2019; Chen et al., 2023; Cian et al., 2017; Danišová et al., 2022; Li et al., 2019; Naguib et al., 2022; Nemati et al., 2021; Onder et al., 2021; Rauff-Adedotun et al., 2023; Rudzińska et al., 2022; Udonsom et al., 2018). Moreover, a meta-analysis highlighted that in certain countries in Southeast Asia, the prevalence can reach 100%, depending on the animal host (Rauff-Adedotun et al., 2020). Revealing a significant variability in the prevalence of the protists at both national and global levels.

This is the first study to detect higher frequency of *Blastocystis* in companion animals such as dogs and cats in Colombia (Supplementary Table 2), as is being reported domestic animals in shelter environment are in higher risk of carrying enteropathogens (Ruaux and Stang, 2014), we hypothesized that dogs residing in rural environments, where the likelihood contact with diverse animal species, might be a contributing factor to increase the prevalence of *Blastocystis* in dogs. Previous research have primarily focused on investigating the presence of the microeukaryote and the potential associations between dogs and urban settings, including their owners infection (Mahdavi et al., 2022; Paulos et al., 2018; Potes-Morales and Crespo-Ortiz, 2023; Ruaux and Stang, 2014; Rudzińska et al., 2022). However, there remains insufficient studies addressing the prevalence of *Blastocystis* in dogs residing in rural areas.

While the initial screening identified a high frequency of *Blastocystis* in dog samples, there was a specific challenge attempting to amplify the complete 18S gene. Despite, the inability to perform complete gene sequencing, the preliminary data obtained from partial gene amplification was able to identify a high prevalence of the infection within the dog population. This shows that partial gene amplification can serve as an early diagnostic tool for veterinarians, researchers, and surveillance efforts. Further research and continued surveillance are needed to explore the complex epidemiology of *Blastocystis* and its potential impact on companion animals and human populations.

Although *Blastocystis* exhibits a substantial worldwide prevalence in cattle (Cabrine-Santos et al., 2021; Higuera et al., 2021; Mokhtar and Youssef, 2018; Naguib et al., 2022; Udonsom et al., 2018), our study showed no positive results in this animal samples. This discrepancy may be attributed to various factors. Firstly, the methodology employed, including diagnosis criteria, sample selection and the chosen location from a specific geographic region of Colombia and specific point on time, may have played a role in this results. Moreover, cattle conditions can vary and contribute to variable prevalences, the unique characteristics of the cattle population and regional differences, might influence in the absence of positive cattle samples. Furthermore, gut environment

and microbiota composition of cattle are complex and dynamic, due to factors such as diet, and breed. To the best of our knowledge, all of the animals included in this study have healthy conditions. However, the overall health and immune status and management practices can impact in the susceptibility of enteric microorganism (Xu et al., 2021). The partial differences between the local experimental context and global perspective should be approach with a more extensive dataset. This could help understanding the complex interplay of factors that influenced the outcomes of our study and contribute to a more comprehensive understanding of *Blastocystis* prevalence in cattle.

Significantly, the current analysis revealed differences in subtype distribution among hosts and regions. *Blastocystis* has been identified in human samples throughout the Americas, Europe, Africa and Asia, these molecular studies have reported the presence of commonly isolated STs in humans including ST1-ST4 (Ahmed et al., 2022; Ajjampur and Tan, 2016; Gabrielli et al., 2020; Jiménez et al., 2022, 2019; Munsaka et al., 2022; Poulsen et al., 2016; Stensvold et al., 2020). In Colombia, subtypes ST1 to ST3 were identified previously. Particularly, ST2 in the Bolivar region (Higuera et al., 2020b), which was exclusively detected in human samples from Bolivar in our study, suggesting a potential host-specific preference for this subtype and regional variation in *Blastocystis* prevalence and subtype frequency (Figure 3b) (Stensvold and Clark, 2020). In contrast, several subtypes (ST5, ST10, ST14, ST15, ST21, ST24, ST25 and ST26) were mainly found in animal samples. The analysis of samples from animals revealed a higher variety of *Blastocystis* subtypes in comparison to the human samples (Supplementary Table 3). This subtype variety may occurred due to various factors, such as interactions between different animal species, their environments, and their immune responses to the microorganism (Alzate et al., 2020; Deng et al., 2023, 2021; Iebba et al., 2016; Rojas-Velázquez et al., 2022; Yañez et al., 2021). Notably, our results revealed an absence of overlapping *Blastocystis* subtypes between different animal species, such as pigs and sheep (Supplementary Table 1) (Supplementary Figure 1). Instead, distinct subtypes were observed in each group, suggesting unique subtype assignments for each animal category (Figure 3a). This pattern may suggest that certain *Blastocystis* subtypes have a specific host tendency, potentially pointing towards divergent transmission routes for these subtypes among animals within the same geographical area (Deng et al., 2021; Jinatham et al., 2021).

In addition to the above results, *Blastocystis* subtyping detected a total of four different subtypes in pig samples, among which ST5 and ST15 were the most common (Figure 4a). The high prevalence of *Blastocystis* in pigs has been previously associated with host

factors such as immune system status, gender, and age, the latter being the principal property which had been related to its prevalence (Hublin et al., 2020). Remarkably, ST15 was detected for the first time in Colombia during this study. It has been suggested that ST15 has a mammalian origin, the elevated detection of ST15 in pig feces supports this hypothesis (Danišová and Valenčáková, 2021; Wylezich et al., 2019), and previously reported in camels, buffalo, wild boars and gibbons (Asghari et al., 2021; Pawestri et al., 2022; Russini et al., 2020), as it has been identified most in domesticated animals and livestock, which are in close proximity to humans; however, its presence has not been confirmed in humans, further research should be needed to determine its potential for zoonotic transmission (Supplementary Table 4). The identification of subtypes, such as ST15 in animals, highlights the use of Next-Generation Sequencing (NGS) technology to explore the diversity of *Blastocystis* subtypes, the importance of continuous surveillance to monitor the emergence of undetected subtypes and their potential implications for public health.

Blastocystis had been detected in a wide host range including mammals, birds, reptiles and mollusks worldwide (Supplementary table 4), this broad host range suggest that animals might serve as reservoirs of the microeukaryote, the contact with infected animals, their feces or contaminated environment had been linked to human transmission (Hernández et al., 2023; Rudzińska and Sikorska, 2023; Stensvold and Clark, 2016). For instance, livestock, pets and wildlife animals which has close contact with humans such as keepers, owners, veterinarians or farmers, along with contact with other animal species may play an important role in the transmission and serve as potential reservoir for other domestic animals and humans infections (Naguib et al., 2022; Salehi et al., 2022). In this study companion pets such as cats and dogs showed a moderate frequency of *Blastocystis*, this proximity could increase the risk of zoonotic transmission, particularly if pets carry possible zoonotic subtypes. Recently, Hernández et al. demonstrated variable associations between *Blastocystis* infection in Colombian schoolchildren and contact with domestic animals. Comparisons between infection status and animal exposure revealed increased odds of ST1 and mixed subtype infections with dogs and cats living in the same house. Conversely, the presence of chickens was associated with decreased odds for multiple *Blastocystis* infection outcomes. The dichotomous risks observed with companion animals compared to chickens highlight that animal reservoirs and zoonotic transmission dynamics of *Blastocystis* can be complex (Hernández et al., 2023). Additionally, considering companion animal owners can harbor *Blastocystis* without showing clinical signs, potentially exposing other humans or animals to the protist. Further investigations

utilizing molecular epidemiology approaches are warranted to clarify potential zoonotic and animal-human transmission routes for this common intestinal parasite. Elucidating the relationships between *Blastocystis* genetic diversity across host species will provide crucial insights into its public health significance. The detection of *Blastocystis* in diverse animal species indicates the potential for zoonotic transmission and the need for further investigation to understand the transmission dynamics between animals and humans (Maloney et al., 2021a).

Among the animal hosts, sheep exhibited the highest diversity of *Blastocystis* subtypes, with seven different subtypes identified which correspond to previously reported subtypes in the same region (Higuera et al., 2021) (Supplementary Table 4). A considerable proportion of sheep samples showed evidence of mixed infections, suggesting a tendency for co-infection with multiple *Blastocystis* subtypes within individual hosts. Moreover, mixed infections were observed in some human samples, with co-occurrence of two subtypes (ST1 and ST3) although less frequently compared to animals (Figure 4). The presence of multiple subtypes in a single host may be influenced by various factors, including alterations in the gut microbiota, which can involve the presence of diverse bacterial species and changes in the microenvironment (Vega et al., 2021; Vega Romero, 2020). Host immune responses, as well as factors related to food and water supplies, also likely contribute to shaping the diversity of microorganisms, including *Blastocystis* (Deng et al., 2023; Jinatham et al., 2021). Socioeconomic factors, such as poverty or inadequate living conditions, may further contribute to the acquisition of gut parasitism, as individuals may be more exposed to such infections (Higuera et al., 2020b). These findings suggest possible interactions and competition between *Blastocystis* subtypes (Supplementary Figure 1). However, further studies are required to determine whether these interactions follow specific patterns that facilitate the coexistence of multiple subtypes within a single host. Knowledge of these mechanisms may lead to a better understanding on the maintenance and prevalence of *Blastocystis* subtypes in animal populations.

In the present study we identified the presence of *Blastocystis* subtypes ST1 and ST3 in both human and animal hosts. These subtypes have previously been linked to gastrointestinal symptoms such as diarrhea, abdominal pain, and urticaria in humans, and there have been suggestions of an association with Inflammatory Bowel Disease (IBD) (Peña et al., 2020, 2019). Interestingly, we observed the presence of these subtypes in pigs and a monkey from the zoo, which are in regular contact with their keepers, farmers in the case of pigs, and zookeepers in the case of the monkey (Supplementary Table 2). This close human-animal interaction poses a potential risk of

zoonotic transmission, whereby *Blastocystis* may be transmitted between animals and humans(Asghari et al., 2021; Cian et al., 2017; Naguib et al., 2022; Rauff-Adedotun et al., 2023; Stensvold and Clark, 2020). Such transmission may result in the dissemination of pathogenic subtypes and could have implications for public health and the welfare of both animals and humans.

While this study provides valuable information on *Blastocystis* frequency and subtype distribution in some regions from Colombia, it also has several limitations that should be considered. Firstly, the used samples may not represent the entire population of humans and animal species in the sampled regions, even though is informative, a larger sample size would enhance statistical robustness. Moreover, due to the cross-sectional design, this study shows the prevalence of *Blastocystis* on a specific point in time which make it impossible to track the changes of the microeukaryote over the time, demanding further longitudinal investigations. Furthermore, this study lacks detailed clinical data form human hosts restricting the understanding of potential associations between the subtypes and clinical symptoms. Although this study suggests the potential for zoonotic transmission of *Blastocystis*, it does not directly investigate transmission dynamics between animals and humans or public health risks; additional investigation including contact tracing and extend human health impact is critical for confirmation of transmission and exploring factors influencing health implications. Furthermore, in this study is important to consider the relatively high error rate introduced in sequences using nanopore technology, which can be attributed to base-calling inaccuracies, overestimated quality scores, errors in homopolymeric or short repeat regions (Delahaye and Nicolas, 2021; Sahlin and Medvedev, 2021), it is crucial to recognize that despite of ensuring updated basecaller and executing manual base-calling algorithm to improve accuracy and mitigate the impact in the obtained results, this technology continues to be improved and may introduce errors.

5. Conclusion

Finally, the present study demonstrates that *Blastocystis* is prevalent in both humans and animals, and the use of NGS allows for the identification of a variety of subtypes, showing both inter and intra-subtype variation. The implementation of Next-Generation Sequencing (NGS) proved instrumental in providing a thorough molecular characterization of *Blastocystis*. The identification of suspected zoonotic subtypes provides valuable information to understand the transmission dynamics of the parasite and potential role of animals as reservoirs for *Blastocystis* subtypes. However, future studies should consider other regions of the country, different domestic and wild animal

species, and variables describing the health status of the individuals to determine potential influences on parasite presence. Additionally, establishing relationships between humans and animals in contact with each other is crucial for a comprehensive understanding of *Blastocystis* transmission pathways. A One Health approach, involving collaboration between human and animal health sectors, is essential to address the potential public health implications of *Blastocystis* transmission and establish effective preventive strategies.

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8. Supplementary Tables

Supplementary Table 1. DNA Concentration of samples across Colombian Departments.

Sample Name	Region	Deparmet	Localization	Sample Type	Nucleic Acid Conc.	Unit	A260	A280	260/280	260/230	Quantification Date	Barcode
CAS-003	Orinoquía	**Casanare**	Pore	DNA	735,7	ng/µl	14,714	7,023	2,1	2,33	02/02/2022 15:03:03	45
CAS-004	Orinoquía	**Casanare**	Pore	DNA	682,5	ng/µl	13,65	6,506	2,1	2,41	02/02/2022 15:04:18	46
BOL-035	Caribe	**Bolívar**	Mompos	DNA	562,5	ng/µl	11,249	5,49	2,05	2,27	02/02/2022 14:57:25	47
CAS-016	Orinoquía	**Casanare**	Pore	DNA	546,3	ng/µl	10,927	5,27	2,07	2,37	02/02/2022 15:12:49	NA
CAS-017	Orinoquía	**Casanare**	Pore	DNA	535,5	ng/µl	10,71	5,171	2,07	2,32	02/02/2022 15:13:40	48
CAS-005	Orinoquía	**Casanare**	Pore	DNA	506,1	ng/µl	10,121	4,882	2,07	2,27	02/02/2022 15:05:07	49
CAS-033	Orinoquía	**Casanare**	Pore	DNA	489,8	ng/µl	9,796	5,108	1,92	1,94	02/02/2022 15:17:20	50
CAS-015	Orinoquía	**Casanare**	Pore	DNA	481,1	ng/µl	9,621	4,593	2,09	2,36	02/02/2022 15:11:55	51
CAS-012	Orinoquía	**Casanare**	Pore	DNA	441,3	ng/µl	8,827	4,215	2,09	2,37	02/02/2022 15:10:17	52
BOL-011	Caribe	**Bolívar**	Mompos	DNA	419	ng/µl	8,38	5,74	1,46	1,39	02/02/2022 14:28:20	53
CAS-041	Orinoquía	**Casanare**	Yopal	DNA	385,9	ng/µl	7,717	3,927	1,97	1,98	02/02/2022 15:20:05	54
BOL-036	Caribe	**Bolívar**	Mompos	DNA	376,4	ng/µl	7,528	3,82	1,97	2,01	02/02/2022 14:58:30	55
CAS-053	Orinoquía	**Casanare**	Tamara	DNA	375,5	ng/µl	7,51	3,801	1,98	1,82	02/02/2022 15:25:29	56

CAS-001	Orinoquía	**Casanare**	Pore	DNA	374,9	ng/µl	7,499	3,638	2,06	2,27	02/02/2022 15:02:01	57
BOL-030	Caribe	**Bolívar**	Mompos	DNA	368,1	ng/µl	7,361	3,535	2,08	2,15	02/02/2022 14:44:12	58
CAS-006	Orinoquía	**Casanare**	Pore	DNA	355,8	ng/µl	7,116	3,414	2,08	2,25	02/02/2022 15:05:55	59
BOL-027	Caribe	**Bolívar**	Mompos	DNA	345,9	ng/µl	6,919	3,47	1,99	2,05	02/02/2022 14:52:09	60
CAS-007	Orinoquía	**Casanare**	Pore	DNA	334,5	ng/µl	6,691	3,2	2,09	2,3	02/02/2022 15:06:51	61
BOL-016	Caribe	**Bolívar**	Mompos	DNA	332,5	ng/µl	6,651	3,219	2,07	2,16	02/02/2022 14:41:22	62
BOL-013	Caribe	**Bolívar**	Mompos	DNA	330,2	ng/µl	6,603	3,417	1,93	1,64	02/02/2022 14:38:13	63
CAS-051	Orinoquía	**Casanare**	Tamara	DNA	325,7	ng/µl	6,514	3,33	1,96	1,97	02/02/2022 15:24:33	64
CAS-035	Orinoquía	**Casanare**	Pore	DNA	303	ng/µl	6,061	3,062	1,98	2,04	02/02/2022 15:18:53	65
BOL-022	Caribe	**Bolívar**	Mompos	DNA	291,7	ng/µl	5,834	2,86	2,04	2,31	02/02/2022 14:46:08	66
CAS-042	Orinoquía	**Casanare**	Yopal	DNA	285,8	ng/µl	5,715	2,92	1,96	2,1	02/02/2022 15:20:50	67
CAS-043	Orinoquía	**Casanare**	Yopal	DNA	259,9	ng/µl	5,198	2,615	1,99	1,99	02/02/2022 15:21:34	68
CAS-034	Orinoquía	**Casanare**	Pore	DNA	257,4	ng/µl	5,149	2,691	1,91	1,7	02/02/2022 15:18:16	69
BOL-026	Caribe	**Bolívar**	Mompos	DNA	255,1	ng/µl	5,101	2,573	1,98	2,12	02/02/2022 14:51:12	70
BOL-029	Caribe	**Bolívar**	Mompos	DNA	253,4	ng/µl	5,068	2,642	1,92	1,85	02/02/2022 14:52:59	71
BOL-010	Caribe	**Bolívar**	Mompos	DNA	251,4	ng/µl	5,029	2,413	2,08	2,22	02/02/2022 14:25:57	72
CAS-050	Orinoquía	**Casanare**	Tamara	DNA	247,9	ng/µl	4,958	2,504	1,98	1,94	02/02/2022 15:23:50	73
BOL-012	Caribe	**Bolívar**	Mompos	DNA	228,3	ng/µl	4,567	2,187	2,09	2,4	02/02/2022 14:37:08	NA

BOL-024	Caribe	**Bolívar**	Mompos	DNA	219,2	ng/µl	4,384	2,13	2,06	2,28	02/02/2022 14:47:54	NA
BOL-015	Caribe	**Bolívar**	Mompos	DNA	216,8	ng/µl	4,336	2,113	2,05	2,19	02/02/2022 14:40:28	NA
BOL-025	Caribe	**Bolívar**	Mompos	DNA	210,9	ng/µl	4,218	2,155	1,96	1,61	02/02/2022 14:48:51	NA
CAS-045	Orinoquía	**Casanare**	Yopal	DNA	206,3	ng/µl	4,125	2,065	2	2,04	02/02/2022 15:22:27	NA
BOL-023	Caribe	**Bolívar**	Mompos	DNA	204,2	ng/µl	4,084	2,047	1,99	2,07	02/02/2022 14:46:59	NA
BOL-014	Caribe	**Bolívar**	Mompos	DNA	196,3	ng/µl	3,926	1,959	2	2,29	02/02/2022 14:39:09	NA
BOL-017	Caribe	**Bolívar**	Mompos	DNA	194,2	ng/µl	3,885	1,913	2,03	2,22	02/02/2022 14:42:17	NA
BOL-032	Caribe	**Bolívar**	Mompos	DNA	188,8	ng/µl	3,776	1,86	2,03	2,2	02/02/2022 14:56:14	NA
CAS-047	Orinoquía	**Casanare**	Yopal	DNA	187,4	ng/µl	3,749	1,916	1,96	1,94	02/02/2022 15:23:10	NA
BOL-019	Caribe	**Bolívar**	Mompos	DNA	174,6	ng/µl	3,492	1,811	1,93	1,91	02/02/2022 14:43:18	NA
CAS-014	Orinoquía	**Casanare**	Pore	DNA	143,2	ng/µl	2,864	1,371	2,09	2,52	02/02/2022 15:11:04	NA
BOL-020	Caribe	**Bolívar**	Mompos	DNA	142,5	ng/µl	2,849	1,41	2,02	2,49	02/02/2022 14:53:56	NA
BOL-021	Caribe	**Bolívar**	Mompos	DNA	138,5	ng/µl	2,771	1,392	1,99	2,08	02/02/2022 14:45:08	NA
CAS-030	Orinoquía	**Casanare**	Pore	DNA	138,1	ng/µl	2,762	1,466	1,88	2,18	02/02/2022 15:16:39	NA
CAS-010	Orinoquía	**Casanare**	Pore	DNA	134,6	ng/µl	2,692	1,269	2,12	2,44	02/02/2022 15:09:16	NA
CAS-018	Orinoquía	**Casanare**	Pore	DNA	115,5	ng/µl	2,31	1,131	2,04	2,3	02/02/2022 15:14:35	NA
BOL-037	Caribe	**Bolívar**	Mompos	DNA	107,7	ng/µl	2,154	1,097	1,96	2,11	02/02/2022 14:59:26	NA
CAS-009	Orinoquía	**Casanare**	Pore	DNA	93,1	ng/µl	1,863	0,904	2,06	2,55	02/02/2022 15:08:28	NA

BOL-031	Caribe	**Bolívar**	Mompos	DNA	82,7	ng/µl	1,653	0,826	2	2,17	02/02/2022 14:55:07	NA
CAS-008	Orinoquía	**Casanare**	Pore	DNA	79,7	ng/µl	1,594	0,868	1,84	1,53	02/02/2022 15:07:38	NA
107	Pacífico	**Cauca**	Popayán	DNA	79,4	ng/µl	1,587	0,912	1,74	0,58	02/02/2022 10:13:54	NA
105	Pacífico	**Cauca**	Popayán	DNA	44,9	ng/µl	0,897	0,528	1,7	0,6	02/02/2022 10:12:37	NA
211	Pacífico	**Cauca**	Popayán	DNA	27,6	ng/µl	0,551	0,373	1,48	0,6	02/02/2022 14:32:16	NA
21	Pacífico	**Cauca**	Popayán	DNA	24,4	ng/µl	0,488	0,323	1,51	0,4	02/02/2022 9:58:53	NA
4	Pacífico	**Cauca**	Popayán	DNA	19,9	ng/µl	0,398	0,184	2,16	0,78	02/02/2022 9:55:18	NA
148	Pacífico	**Cauca**	Popayán	DNA	17,7	ng/µl	0,354	0,216	1,64	0,5	02/02/2022 10:19:02	NA
CAS-027	Orinoquía	**Casanare**	Pore	DNA	16,6	ng/µl	0,332	0,165	2,01	3,35	02/02/2022 15:15:25	NA
156	Pacífico	**Cauca**	Popayán	DNA	14,4	ng/µl	0,288	0,175	1,64	0,41	09/02/2022 10:16:33	NA
132	Pacífico	**Cauca**	Popayán	DNA	14,3	ng/µl	0,286	0,163	1,75	0,25	02/02/2022 10:17:54	NA
262	Pacífico	**Cauca**	Popayán	DNA	12,8	ng/µl	0,255	0,192	1,33	0,55	02/02/2022 14:36:07	NA
157	Pacífico	**Cauca**	Popayán	DNA	12,5	ng/µl	0,249	0,191	1,3	0,75	02/02/2022 14:22:39	NA
97	Pacífico	**Cauca**	Popayán	DNA	11,7	ng/µl	0,235	0,134	1,75	0,61	02/02/2022 10:08:37	NA
167	Pacífico	**Cauca**	Popayán	DNA	10,3	ng/µl	0,206	0,167	1,24	0,44	09/02/2022 10:23:27	NA
103	Pacífico	**Cauca**	Popayán	DNA	8,1	ng/µl	0,161	0,101	1,61	0,36	02/02/2022 10:10:22	NA
188	Pacífico	**Cauca**	Popayán	DNA	8,1	ng/µl	0,162	0,117	1,38	0,27	02/02/2022 14:23:29	NA
66	Pacífico	**Cauca**	Popayán	DNA	7,7	ng/µl	0,154	0,052	2,94	0,17	02/02/2022 10:04:26	NA
257	Pacífico	**Cauca**	Popayán	DNA	7	ng/µl	0,14	0,067	2,1	3,27	02/02/2022 14:34:31	NA

109	Pacífico	**Cauca**	Popayán	DNA	6,5	ng/µl	0,131	0,074	1,77	0,41	09/02/2022 10:26:31	NA
200	Pacífico	**Cauca**	Popayán	DNA	6,3	ng/µl	0,125	0,078	1,6	0,38	02/02/2022 14:30:55	NA
45	Pacífico	**Cauca**	Popayán	DNA	6,3	ng/µl	0,126	0,036	3,53	0,16	02/02/2022 10:00:57	NA
199	Pacífico	**Cauca**	Popayán	DNA	6,2	ng/µl	0,123	0,056	2,21	0,42	09/02/2022 10:26:10	NA
198	Pacífico	**Cauca**	Popayán	DNA	5,9	ng/µl	0,117	0,062	1,89	0,26	02/02/2022 14:29:37	NA
121	Pacífico	**Cauca**	Popayán	DNA	4,9	ng/µl	0,098	0,052	1,88	0,25	02/02/2022 10:16:27	NA
125	Pacífico	**Cauca**	Popayán	DNA	4,8	ng/µl	0,097	0,058	1,66	0,56	09/02/2022 12:31:53	NA
223	Pacífico	**Cauca**	Popayán	DNA	4,7	ng/µl	0,094	0,035	2,64	0,78	09/02/2022 12:41:17	NA
123	Pacífico	**Cauca**	Popayán	DNA	4,6	ng/µl	0,093	0,049	1,91	0,48	09/02/2022 10:18:26	NA
52	Pacífico	**Cauca**	Popayán	DNA	4,6	ng/µl	0,092	0,01	8,98	0,11	02/02/2022 10:03:40	NA
79	Pacífico	**Cauca**	Popayán	DNA	4,5	ng/µl	0,091	0,015	5,88	0,13	02/02/2022 10:05:22	NA
50	Pacífico	**Cauca**	Popayán	DNA	4,3	ng/µl	0,087	0,019	4,67	0,1	02/02/2022 10:02:51	NA
10	Pacífico	**Cauca**	Popayán	DNA	4,1	ng/µl	0,081	0,028	2,93	0,41	02/02/2022 9:56:33	NA
177	Pacífico	**Cauca**	Popayán	DNA	4,1	ng/µl	0,082	0,061	1,34	0,26	09/02/2022 12:43:43	NA
214	Pacífico	**Cauca**	Popayán	DNA	4	ng/µl	0,08	0,043	1,88	0,25	02/02/2022 14:33:25	NA
197	Pacífico	**Cauca**	Popayán	DNA	3,9	ng/µl	0,079	0,046	1,71	0,37	02/02/2022 14:24:36	NA
17	Pacífico	**Cauca**	Popayán	DNA	3,8	ng/µl	0,075	0,042	1,79	0,44	09/02/2022 10:20:27	NA
183	Pacífico	**Cauca**	Popayán	DNA	3,6	ng/µl	0,073	0,038	1,91	0,64	09/02/2022 12:34:14	NA

101	Pacífico	**Cauca**	Popayán	DNA	3,2	ng/µl	0,065	0,043	1,52	0,51	09/02/2022 12:35:41	NA
215	Pacífico	**Cauca**	Popayán	DNA	3,2	ng/µl	0,065	0,046	1,41	0,56	09/02/2022 12:29:18	NA
265	Pacífico	**Cauca**	Popayán	DNA	3	ng/µl	0,059	0,037	1,58	0,42	09/02/2022 10:21:26	NA
96	Pacífico	**Cauca**	Popayán	DNA	2,5	ng/µl	0,05	0,022	2,26	0,73	02/02/2022 10:07:33	NA
195	Pacífico	**Cauca**	Popayán	DNA	2,4	ng/µl	0,047	0,028	1,71	0,43	09/02/2022 12:28:14	NA
112	Pacífico	**Cauca**	Popayán	DNA	2,3	ng/µl	0,047	0,027	1,75	0,47	09/02/2022 10:22:20	NA
133	Pacífico	**Cauca**	Popayán	DNA	2,1	ng/µl	0,041	0,028	1,47	0,37	09/02/2022 10:17:38	NA
248	Pacífico	**Cauca**	Popayán	DNA	2	ng/µl	0,04	0,006	7,2	-13,18	09/02/2022 12:44:42	NA
18	Pacífico	**Cauca**	Popayán	DNA	1,8	ng/µl	0,036	0,008	4,26	0,28	09/02/2022 12:40:33	NA
202	Pacífico	**Cauca**	Popayán	DNA	1,7	ng/µl	0,034	0,025	1,37	0,45	09/02/2022 10:24:22	NA
221	Pacífico	**Cauca**	Popayán	DNA	1,7	ng/µl	0,035	0,016	2,13	0,87	09/02/2022 12:39:09	NA
39	Pacífico	**Cauca**	Popayán	DNA	1,5	ng/µl	0,03	0,004	7,72	-1,29	09/02/2022 12:23:13	NA
99	Pacífico	**Cauca**	Popayán	DNA	1,5	ng/µl	0,03	0,006	4,85	-0,89	09/02/2022 12:42:43	NA
127	Pacífico	**Cauca**	Popayán	DNA	1,4	ng/µl	0,029	0,028	1,04	17,27	09/02/2022 12:32:55	NA
159	Pacífico	**Cauca**	Popayán	DNA	1,4	ng/µl	0,027	0,018	1,53	0,29	09/02/2022 10:25:30	NA
260	Pacífico	**Cauca**	Popayán	DNA	1,4	ng/µl	0,028	0,007	3,83	0,3	09/02/2022 10:19:33	NA
95	Pacífico	**Cauca**	Popayán	DNA	1,4	ng/µl	0,027	0,006	4,41	0,21	02/02/2022 10:06:12	NA
56	Pacífico	**Cauca**	Popayán	DNA	1,3	ng/µl	0,026	0,011	2,39	0,15	09/02/2022 10:15:38	NA

110	Pacífico	**Cauca**	Popayán	DNA	1,2	ng/µl	0,025	0,013	1,87	-1,16	09/02/2022 12:38:02	NA
152	Pacífico	**Cauca**	Popayán	DNA	1,2	ng/µl	0,024	0,016	1,51	0,38	09/02/2022 10:28:35	NA
244	Pacífico	**Cauca**	Popayán	DNA	1,2	ng/µl	0,023	0,002	10,41	-0,84	09/02/2022 12:36:49	NA
30	Pacífico	**Cauca**	Popayán	DNA	1,2	ng/µl	0,024	0,012	1,99	-6,63	09/02/2022 12:30:37	NA
270	Pacífico	**Cauca**	Popayán	DNA	1,1	ng/µl	0,023	0,01	2,2	-0,87	09/02/2022 12:24:53	NA
14	Pacífico	**Cauca**	Popayán	DNA	0,6	ng/µl	0,012	0,019	0,67	0,15	02/02/2022 9:57:21	NA
37	Pacífico	**Cauca**	Popayán	DNA	0,6	ng/µl	0,011	-0,006	-1,78	-0,23	09/02/2022 12:22:07	NA

Supplementary Table 2. Data Summary from animal samples.

Location	Animal	Number of samples collected	Type of Sample	Number of positive samples by PCR (%)	Number of positive samples by Long Amplicon PCR (%)	Number of samples ST assignation (%)	Number of samples with mixed infection	Subtypes detected
Bogotá	Cat (*Felis catus*)	39	Individual	11(28.2%)	0	0	NA	NA
Boyacá	Cat (*Felis catus*)	57	Individual	21(36.8%)	0	0	NA	NA
Boyacá	Dog (*Canis lupus familiaris*)	35	Individual	22(62.8%)	0	0	NA	NA
Boyacá	Domestic pig (*Sus scrofa domesticus*)	36	Individual	19(52.7%)	15(78.9%)	14/15 (93.3%)	9/14 (64.28%)	ST1, ST3, ST5, ST15
Boyacá	Cattle (*Bos taurus*)	29	Individual	0(0%)	0	0	NA	NA
Boyacá	Goat (*Capra aegagrus hircus*)	20	Individual	0(0%)	0	0	NA	NA
Boyacá	Sheep (*Ovis aries*)	38	Individual	11(28,9%)	7(63.6%)	7(100%)	6/7 (85.71%)	ST10, ST14, ST21, ST24, ST25, ST26
Boyacá	Greater grison (*Galictis vittata*)	1	Individual	0(0%)	0	0	NA	NA
Boyacá	Cougar (*Puma concolor*)	1	Pool	0(0%)	0	0	NA	NA
Boyacá	Jaguar (*Panthera onca*)	1	Pool	0(0%)	0	0	NA	NA

Boyacá	Crab-eating fox/ Forest fox (*Cerdocyon thous*)	1	Pool	0(0%)	0	0	NA	NA
Boyacá	Grey fox (*Urocyon cinereoargenteus)*	1	Individual	0(0%)	0	0	NA	NA
Boyacá	South America coati (*Nasua nasua*)	1	Pool	0(0%)	0	0	NA	NA
Boyacá	Tayra (*Eira barbara*)	1	Pool	0(0%)	0	0	NA	NA
Boyacá	Brown bear (*Ursus arctos*)	1	Pool	0(0%)	0	0	NA	NA
Boyacá	Tiger (*Panthera tigris*)	2	Individual	0(0%)	0	0	NA	NA
Boyacá	Lioness (*Panthera leo*)	1	Pool	0(0%)	0	0	NA	NA
Boyacá	Lion (*Panthera leo*)	1	Pool	0(0%)	0	0	NA	NA
Boyacá	White-tailed deer (*Odocoileus virginianus*)	1	Pool	1(100%)	0	0	NA	NA
Boyacá	Tufted capuchin (*Sapajus apella*)	1	Pool	0(0%)	0	0	NA	NA
Boyacá	Brown woolly monkey (*Lagothrix lagotrichia*)	1	Pool	0(0%)	0	0	NA	NA
Boyacá	Black agouti (*Dasyprocta fuliginosa*)	1	Pool	1(100%)	0	0	NA	NA
Boyacá	Lowland/Spotted paca (*Cuniculus paca*)	1	Pool	0(0%)	0	0	NA	NA
Boyacá	Capybara (*Hydrochoerus hydrochaeris*)	1	Pool	0(0%)	0	0	NA	NA

Boyacá	Guianan squirrel monkey (*Saimiri sciureus*)	1	Pool	0(0%)	0	0	NA	NA
Boyacá	Domestic pig (*Sus scrofa domesticus*)	1	Pool	0(0%)	0	0	NA	NA
Boyacá	Kinkajou (*Potos flavus*)	1	Pool	0(0%)	0	0	NA	NA
Boyacá	Bison (*Bison bison*)	1	Pool	0(0%)	0	0	NA	NA
Boyacá	Brown spider monkey (*Ateles hybridus*)	1	Pool	1(100%)	1(100%)	1(100%)	0	ST1
	Total	277		87 (31.4%)	23 (26.43%)	22 (95.65%)	15 (68.18%)	10 STs

Supplementary Table 3. Genetic diversity indices of *Blastocystis* by hosts.

	n	**π**	**Θ**	***S***	***h***	**Hd**	**SD**	**D*T***	**Fu and Li's D**	**Fu and Li's F**	**R2**
Humans	3	0,14011	0,14824	333	3	1	0,272	NA	NA	NA	0,1235
Sheeps	6	0,09301	0,11466	355	6	1	0,096	-1,22892	-0,91094	-1,07749	0,1357
Pigs	2	0,14502	0,14502	249	2	1	0,5	NA	NA	NA	0,5
Total	11	0,11432	0,15782	530	11	1	0,039	-1,33788	-1,09049	-1,3118	0,1054

n: number of samples, π: average pairwise distance, Θ: population mutation rate, *S*: number of segregating sites, *h*: number of haplotypes, Hd: Haplotype diversity, SD: Standard deviation of haplotype diversity, D*T*: Tajima´s D, Fu and Li´s D & F, R2: Ramos-Onsins & Rozas´R2

Supplementary Table 4. Summary of *Blastocystis* subtypes reported in different animal species.

Class	Order	Host	Location	STs	Reference
Aves	Anseriformes	*Anas* spp (Garganey & Wild Duck)	Brasil, Malaysia, Egypt, Thailand	ST1, ST2, ST7, ST10, ST27, ST28	(Maloney et al., 2020; Mokhtar and Youssef, 2018; Noradilah et al., 2017; Tantrawatpan et al., 2023)
Aves	Anseriformes	*Anser* spp (Swan Goose)	Brasil, Malaysia, Egypt, Thailand	ST1, ST2, ST3, ST6, ST7	(Barbosa et al., 2017; Maloney et al., 2020; Mokhtar and Youssef, 2018; Noradilah et al., 2017; Tantrawatpan et al., 2023)
Aves	Anseriformes	*Cairina moschata momelanotus* (Muscovy Duck)	Brasil	ST7, ST10	(Maloney et al., 2020)
Aves	Anseriformes	*Asarcornis scutulata (White-winged duck)*	Thailand	ST7	(Tantrawatpan et al., 2023)
Aves	Casuariiformes	*Casuarius casuarius* (Cassowary)	Australia	ST2	(Roberts et al., 2013)
Aves	Columbiformes	*Columba livia* (Pigeon)	China, Iran, Egypt	ST6, ST7, ST13	(Asghari et al., 2019; Mokhtar and Youssef, 2018; Wang et al., 2018)
Aves	Galliformes	*Gallus domesticus* (Chicken)	Brasil, China, Algeria, Australia, Turkey, Malaysia, Egypt, Thailand	ST2, ST3, ST6, ST7, ST9, ST10, ST14, ST25, ST29	(Boutellis et al., 2021; Maloney et al., 2021a; Mokhtar and Youssef, 2018; Naguib et al., 2022; Onder et al., 2021; Roberts et al., 2013; Tantrawatpan et al., 2023; Wang et al., 2018)
Aves	Galliformes	*Coturnix coturnix* (Quail)	Brasil	ST6, ST7	(Maloney et al., 2020)
Aves	Galliformes	*Gallus gallus* (Red junglefowl)	Brasil	ST5	(Valença-Barbosa et al., 2019)

Aves	Galliformes	*Numida meleagris* (Helmeted Guineafowl)	Brasil	ST6, ST7	(Maloney et al., 2020)
Aves	Galliformes	*Pavo cristatus* (Indian Peafowl)	Brasil, Thailand	ST6, ST7, ST9, ST27, ST28	(Maloney et al., 2020; Tantrawatpan et al., 2023)
Aves	Galliformes	*Phasianus colchicus* (Pheasant)	Brasil	ST6, ST7	(Maloney et al., 2020)
Aves	Galliformes	*Meleagris gallopavo* (Turkey)	Egypt	ST1, ST6, ST7	(Mokhtar and Youssef, 2018)
Aves	Galliformes	*Pavo muticus (Green peafowl)*	Thailand	ST8	(Tantrawatpan et al., 2023)
Aves	Gruiformes	*Grus japonensis* (Red crowned crane)	China	ST6, ST7	(Wang et al., 2018)
Aves	Passeriformes	Corvus cornix (Hooded Crow)	Iran	ST13, ST14	(Asghari et al., 2019)
Aves	Psittaciformes	*Agapornis nigrigenis* (Black-cheeked Lovebird)	Brasil	ST14	(Maloney et al., 2020)
Aves	Struthioniformes	*Struthio camelus* (Ostrich)	Brasil, Australia	ST4, ST5, ST10, ST24	(Maloney et al., 2020; Roberts et al., 2013)
Bivalvia	Ostreida	*Crassostrea virginica* (Eastern Oyster)	México	ST1	(Barbabosa et al., 2021)
Mammalia	Artiodactyla	*Bos taurus* (Cattle)	Brasil, Colombia, USA, Thailand, Turkey, Egypt	ST1, ST3, ST4, ST5, ST10, ST12, ST13, ST14, ST17, ST21, ST23, ST24, ST25, ST26, ST32	(Cabrine-Santos et al., 2021; Fayer et al., 2014; Higuera et al., 2021; Mokhtar and Youssef, 2018; Naguib et al., 2022; Öncü Öner et al., 2022; Onder et al., 2021, 2021; Santin et al., 2011; Tantrawatpan et al., 2023; Udonsom et al., 2018)

Mammalia	Artiodactyla	*Capra hircus* (Goat)	Colombia, Thailand, Malaysia, Egypt	ST1, ST4, ST8, ST10, ST12, ST14, ST21, ST23, ST24, ST25, ST26, ST32	(Higuera et al., 2021; Mokhtar and Youssef, 2018; Noradilah et al., 2017; Tantrawatpan et al., 2023; Udonsom et al., 2018)
Mammalia	Artiodactyla	*Lama glama* (Llama)	Colombia, Algeria	ST10, ST21, ST23, ST24, ST25	(Boutellis et al., 2021; Higuera et al., 2021)
Mammalia	Artiodactyla	*Mazama gouazoubira* (Grayy brocket)	Brasil	ST5	(Oliveira-Arbex et al., 2020)
Mammalia	Artiodactyla	*Sus domesticus* (Pig)	Colombia, Australia, Thailand	ST1, ST3, ST5, ST10, ST12, ST14	(Higuera et al., 2021; Roberts et al., 2013; Tantrawatpan et al., 2023)
Mammalia	Artiodactyla	*Ovis aries* (Sheep)	Colombia, Turkey, Thailand	ST10, ST14, ST21, ST23, ST24, ST26	(Higuera et al., 2021; Onder et al., 2021; Tantrawatpan et al., 2023)
Mammalia	Artiodactyla	*Sus scrofa* (Wild Boar)	Colombia, USA, Brasil	ST1, ST3, ST4, ST5, ST8	(Cabrine-Santos et al., 2021; David et al., 2015; Fayer et al., 2014; Higuera et al., 2021; Santin et al., 2011; Valença-Barbosa et al., 2019)
Mammalia	Artiodactyla	*Odocoileus virginianus* (White-tailed Deer)	USA	ST1, ST3, ST4, ST10, ST14, ST21, ST23, ST24, ST25, ST26, ST30, ST31	(Maloney et al., 2021b)

Mammalia	Artiodactyla	*Cervus elaphus* (Deer)	Australia	ST4	(Roberts et al., 2013)
Mammalia	Artiodactyla	*Giraffa camelopardalis* (Giraffe)	Australia	ST12	(Roberts et al., 2013)
Mammalia	Artiodactyla	*Rangifer tarandus* (Reindeer)	China	ST10, ST13	(Wang et al., 2018)
Mammalia	Artiodactyla	*Cervus nippon* (Sika deer)	China	ST10, ST14	(Wang et al., 2018)
Mammalia	Artiodactyla	Bubalus bubalis (Water Buffalo)	Turkey	ST14	(Onder et al., 2021)
Mammalia	Artiodactyla	*Camelus dromedarius (Camel)*	Egypt	ST1	(Mokhtar and Youssef, 2018)
Mammalia	Artiodactyla	*Muntiacus muntjak* (Common Barking Deer)	Thailand	ST4	(Tantrawatpan et al., 2023)
Mammalia	Artiodactyla	*Bos javabicus* (Banteng)	Thailand	ST14, ST24, ST25, ST26	(Tantrawatpan et al., 2023)
Mammalia	Artiodactyla	*Tragelaphus angasil (Nyala)*	Thailand	ST14, ST24, ST25	(Tantrawatpan et al., 2023)
Mammalia	Carnivora	*Felis catus* (Cat)	USA, Algeria, China, Malaysia, Egypt, Turkey	ST1, ST2, ST3, ST4, ST10, ST14	(Boutellis et al., 2021; Can et al., 2021; Naguib et al., 2022; Noradilah et al., 2017; Ruaux and Stang, 2014)
Mammalia	Carnivora	*Canis lupus familiaris (Dog)*	Colombia, USA, France, Thailand, China, Philippines, Italy, Malaysia	ST1-8, ST10, ST23, ST24	(Gazzonis et al., 2019; Higuera et al., 2021; Li et al., 2019; Noradilah et al., 2017; Osman et al., 2015; Ruaux and Stang, 2014; Udonsom et al., 2018; Villamizar et al., 2019; Wang et al., 2018)
Mammalia	Carnivora	Leopardus tigrinus (Oncilla)	Brasil	ST5	(Oliveira-Arbex et al., 2020)
Mammalia	Carnivora	*Nasua nasua* (South	Brasil	ST1	(Oliveira-Arbex et al., 2020)

		American Coati)			
Mammalia	Carnivora	*Panthera uncia* (Snow Leopard)	Australia	ST4	(Roberts et al., 2013)
Mammalia	Carnivora	*Nyctereutes procyonoides* (Racoon dog)	China	ST3	(Wang et al., 2018)
Mammalia	Carnivora	*Alopex lagopus* (Arctic fox)	China	ST1, ST4, ST7	(Wang et al., 2018)
Mammalia	Cingulata	*Dasypus septemcinctus* (Seven-banded Armadillo)	Brasil	ST8	(Valença-Barbosa et al., 2019)
Mammalia	Didelphimorphia	*Didelphis aurita* (Big-eared Opossum)	Brasil	ST1, ST8	(Valença-Barbosa et al., 2019)
Mammalia	Didelphimorphia	*Metachirus nudicautatus* (Brown Four-eyed Opossum)	Brasil	ST1	(Valença-Barbosa et al., 2019)
Mammalia	Diprotodontia	*Macropus fuliginosus* (Western Grey Kangaroo)	Australia	ST13	(Roberts et al., 2013)
Mammalia	Diprotodontia	*Macropus giganteus* (Eastern Grey Kangaroo)	Australia	ST4	(Roberts et al., 2013)
Mammalia	Diprotodontia	*Macropus rufus* (Red Kangaroo)	Australia	ST4	(Roberts et al., 2013)
Mammalia	Diprotodontia	*Macropus robustus* (Eastern Wallaroo)	Australia	ST4	(Roberts et al., 2013)
Mammalia	Lagomorpha	*Oryctolagus cuniculus* (New Zealand White Rabbit)	China	ST4	(Wang et al., 2018)
Mammalia	Perissodactyla	*Equus ferus caballus (Horse)*	Colombia	ST10, ST14, ST24, ST33, ST34	(Higuera et al., 2021; Maloney et al., 2020)
Mammalia	Perissodactyla	*Equus africanus*	Egypt	ST1	(Mokhtar and Youssef, 2018)

		asinus (Donkeys)			
Mammalia	Primates	*Alouatta spp* (Howler Monkey)	Brasil, Ecuador	ST2, ST3, ST8	(Helenbrook and Whipps, 2021; Oliveira-Arbex et al., 2020; Valença-Barbosa et al., 2019)
Mammalia	Primates	*Aotus* spp (Night Monkey)	Brasil, Perú	ST1, ST8	(Helenbrook and Whipps, 2021; Valença-Barbosa et al., 2019)
Mammalia	Primates	*Ateles* spp (Spider Monkey)	Brasil, Perú	ST2, ST8	(Köster et al., 2022; Oliveira-Arbex et al., 2020; Valença-Barbosa et al., 2019)
Mammalia	Primates	*Callicebus lucifer* (Lucifer titi monkey)	Perú	ST2	(Köster et al., 2022)
Mammalia	Primates	*Lagothrix lagothricha* (Common Woolly Monkey)	Brasil, Perú	ST2, ST3, ST8	(Köster et al., 2022; Oliveira-Arbex et al., 2020; Valença-Barbosa et al., 2019)
Mammalia	Primates	*Macaca mulatta & fuscata (Macaque)*	Brasil, Autralia, Thailand	ST1, ST2, ST3, ST8	(Roberts et al., 2013; Tantrawatpan et al., 2023; Valença-Barbosa et al., 2019)
Mammalia	Primates	*Mandrillus sphinx* (Mandrill)	Brasil	ST3	(Oliveira-Arbex et al., 2020)
Mammalia	Primates	*Pan troglodytes* (Chimpanzee)	Brasil, Australia, Sierra Leone, Côte d'Ivoire	ST1, ST2, ST3, ST11	(Köster et al., 2022; Roberts et al., 2013; Valença-Barbosa et al., 2019)
Mammalia	Primates	*Papio spp* (Baboon)	Brasil, Côte d'Ivoire	ST3	(Oliveira-Arbex et al., 2020; Valença-Barbosa et al., 2019)
Mammalia	Primates	*Pithecia monachus* (Saki Monkey)	Perú	ST2	(Köster et al., 2022)
Mammalia	Primates	*Pongo abelii* (Orang Utan)	Australia	ST2	(Roberts et al., 2013)
Mammalia	Primates	*Gorilla gorilla* (Gorilla)	Australia	ST1, ST2	(Roberts et al., 2013)

Mammalia	Primates	*Trachypithecus francoisi* (Francois Langur)	Australia	ST1	(Roberts et al., 2013)
Mammalia	Primates	*Hylobates lar (Common gibbon)*	Thailand	ST2	(Tantrawatpan et al., 2023)
Mammalia	Proboscidea	*Elephas maximus* (Asian Elephant)	Australia	ST11	(Roberts et al., 2013)
Mammalia	Rodentia	*Heteromyidae* (Rodent)	México	ST4, ST17	(Martinez-Hernandez et al., 2020)
Mammalia	Rodentia	*Hydrochoerus hydrochaeris (Capybara)*	Brasil	ST8, ST1	(Oliveira-Arbex et al., 2020)
Mammalia	Rodentia	*Nectomys squamipes* (South American water rat)	Brasil	ST8	(Valença-Barbosa et al., 2019)
Mammalia	Rodentia	*Rattus rattus* (Black Rat)	Brasil, Malaysia, Thailand	ST3, ST4	(Noradilah et al., 2017; Valença-Barbosa et al., 2019)
Mammalia	Rodentia	*Mus musculus* (Brown Rat)	China	ST4	(Wang et al., 2018)
Mammalia	Rodentia	*Hystrix brachyura* (East Asian Porcupine)	Thailand	ST8	(Tantrawatpan et al., 2023)
Mammalia	Rodentia	*Callosciurus erythraeus* (Pllas's Squirrel)	Thailand	ST4	(Tantrawatpan et al., 2023)
Mammalia	Rodentia	*Callosciurus finlaysonii* (Variable squirrel)	Thailand	ST4	(Tantrawatpan et al., 2023)

9. Supplementary Figures

Supplementary Figure 1. Phylogenetic relationships between the haplotypes from Blastocystis STs sequences. Phylogenetic network (Neighbor-Net) constructed in SplitsTree 5.