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Review

Molecular epidemiology, evolution, and phylogeny of *Entamoeba* spp.

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ABSTRACT

Entamoeba histolytica is a protozoan parasite and the causative agent of amoebiasis in humans. The estimations of the worldwide burden of amoebiasis by the WHO indicated that approximately 500 million people were infected with the parasite and 10% of these individuals had invasive amoebiasis. However, our understanding of the disease burden and epidemiology of human amebiasis has undergone dramatic changes over the last two decades based on molecular analyses. The development of *Entamoeba* genomics has also provided some interesting and valuable information on the evolution and population structure of this parasite. In addition, the use of a number of molecular markers has greatly expanded our understanding of *Entamoeba* host range and genetic diversity. In this review, we re-assessed *Entamoeba* prevalence and species in humans, non-human primates, other animals, and the environment in the context of molecular data. Some issues regarding the evolution and phylogeny of different *Entamoeba* species lineages are also discussed.

1. Introduction

The *Entamoeba* genus contains a group of unicellular, anaerobic, parasitic organisms found in humans, nonhuman primates (NHPs), other vertebrate and invertebrate species worldwide (Ngobeni et al., 2017). Amebiasis, one of the most frequent parasitic disease, contributes towards a heavy burden of diarrhea, especially in developing countries that have poor sanitation in certain regions (Costa, 2018). *Entamoeba histolytica* is classified as a category B priority biodefense pathogen by the National Institute of Allergy and Infectious Diseases (NIAID) (Shirley et al., 2018). To date, there is no vaccine to prevent amebiasis. Although nitroimidazoles are the mainstay treatment for invasive amebiasis, new therapies are still needed because of their toxicity and potential concerns for resistance development (Paulishmiller et al., 2014).

Microscopy has been the most widely used method for identification and assignment of *Entamoeba* organisms to species (Fotedar et al., 2007). Much of the previous literature on amebiasis epidemiology has relied on microscopy as the major diagnostic method, which lacks the ability to differentiate true infection caused by *E. histolytica* from non-pathogenic *Entamoeba* spp. (Turkeltaub et al., 2015). Recently, molecular tools have been increasingly used for the identification, taxonomy, epidemiology, and clinical significance of *Entamoeba* species (Kobayashi et al., 2009; Stensvold et al., 2010b). Small subunit rRNA (SSU rDNA) gene has been widely used to analyze phylogenetic relationships among eukaryotic organisms and detect *Entamoeba* species in stool samples (Stensvold et al., 2011).

The genome sequence of *E. histolytica* was analyzed in 2005, and comparisons with other amoeba genomes has assisted in resolving fundamental issues relating to eukaryote and amoeba phylogeny as well as how LGT (lateral gene transfer) affects the evolution of eukaryotes (Loftus et al., 2005). The ability to rapidly generate whole genome sequences of *Entamoeba* also helped us understand different phenotypes and differential disease causing abilities within and between species (Das and Ganguly, 2014; Weedall and Hall, 2011).

Here, we reviewed published literature that employed molecular detection to re-evaluate the impact of a major gastrointestinal protozoan infection, amebiasis, as a global public health threat. Some tissues about the evolution and phylogeny of different *Entamoeba* species and ribosomal lineages (RLs) have also been included within it.

1.1. Molecular epidemiology of Entamoeba

To date, a number of molecular diagnostic tests are available to assist with diagnosis of intestinal amoebiasis, including conventional PCR, nested PCR, real-time PCR, multiplex PCR, and loop-mediated

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isothermal amplification assay (LAMP) (Khademerfan et al., 2019). For conventional PCR and nested PCR, SSU rDNA, a gene that encodes a 30-kDa protein, DNA highly repetitive sequences, the hemolysin gene HLY6, cysteine proteinase, the serine-rich *E. histolytica* SREHP gene, the actin gene, and tandem repeats in extrachromosomal circular DNA have been targeted to discriminate *Entamoeba* species (Freitas et al., 2004; Zindrou et al., 2001). The real-time PCR assay has been widely used for laboratory diagnosis of infection because it can enhance diagnostic sensitivity, eliminate post-PCR manipulation, and minimize contamination (Fotedar et al., 2007). Moreover, the development of multiplex real time PCR makes it possible to rapidly and simultaneously identify, genotype, and quantify multiple DNA targets in a single reaction (Hamzah et al., 2010). The LAMP assay also has a high sensitivity, specificity, rapidity, and simplicity, and is a good choice for molecular diagnosis, especially for low resource areas (Fernández-Soto et al., 2014). Molecular detection is highly sensitive and specific; however, cost is still a barrier for use as a routine test method in most endemic areas.

1.2. Entamoeba in humans

A total of 110 studies from 47 countries were used to calculate epidemiological figures. The overall molecular prevalence of *Entamoeba* infection was 3.55% (3817/107396) in humans worldwide. Globally, the prevalence ranged from 1.72% (Oceania) to 21.58% (North America) (Fig. 1). In Europe, *Entamoeba* infection is mostly seen in returning travelers or immigrants (Evangelopoulos et al., 2001; Herbinger et al., 2011; ten Hove et al., 2009). In previous studies, the risk for travelers to be infected with *E. histolytica* or *E. dispar* was highest for destinations in West Africa, East Africa, and South and South-East Asia (Herbinger et al., 2011).

In some countries of Asia, Africa, and North America, children and school students seemed to have a higher risk of acquiring amebiasis than the general population (Ali et al., 2003; Efunshile et al., 2015; Matsumura et al., 2019; Oliveira et al., 2015). For example, a school-based cross-sectional survey showed a high rate of *Entamoeba* infection in school children (aged 7–15) in Indonesia, with a prevalence of 52.8% (Matsumura et al., 2019). In previous studies, *E. histolytica* seropositivity was more common in HIV-positive individuals and HIV-infected men who have sex with men and this was supported by molecular data (Beck et al., 2008; Hung et al., 2005; Kobayashi et al., 2017; Nath et al., 2015; Stark et al., 2007).

Entamoeba species that can infect and can be found in the intestinal lumen of humans include *E. histolytica*, *E. dispar*, *E. moshkovskii*, *E. coli*, *E. hartmanni*, *E. polecki*, and *E. bangladeshi* (Ngobeni et al., 2017). *Entamoeba gingivalis* is primarily found in the human oral cavity, but has also been found in the genitourinary tract of intrauterine contraceptive device users in Egypt (Foda and El-Malky, 2012). *Entamoeba nuttalli*, which is prevalent in NHPs, has been detected in a caretaker at a zoo (Levecke et al., 2015). Pathogenic *E. histolytica* and nonpathogenic *E. dispar* represented 81.73% of these infections; the remaining infections were caused by *E. moshkovskii* (10.22%), *E. coli* (1.98%), *E. hartmanni* (0.96%), *E. polecki* (0.04%), *E. gingivalis* (4.58%), and *E. nuttalli* (0.02%). Although local prevalence may significantly vary, *E. dispar* infection is generally much more common than *E. histolytica* worldwide (Fig. 2A).

1.3. Entamoeba in NHPs

The average molecular prevalence of *Entamoeba* infection was 62.88% (3093/4919) in NHPs. Few molecular studies on NHPs have been published to date and most were conducted in Asia, Europe, Africa, and North America (Table S2). *Entamoeba* seems to be more prevalent among NHPs in China (Dong et al., 2017; Feng et al., 2013; Feng et al., 2011; Guan et al., 2016; Wei et al., 2018; Zhang et al., 2019), especially in long-tailed macaques, which had a prevalence of 100% (Feng et al., 2011).

At the molecular level, *Entamoeba* parasites that have been

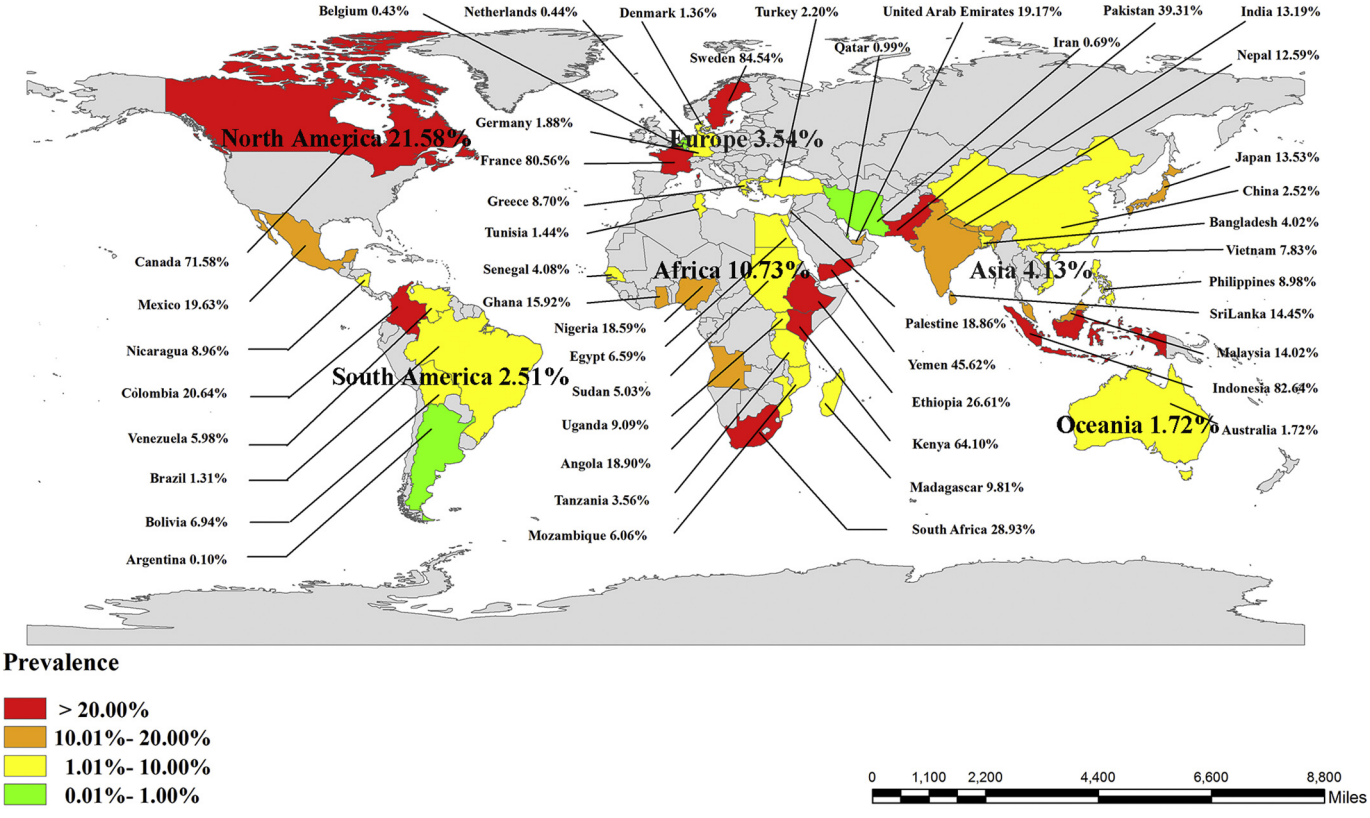

Fig. 1. Prevalence and geographic distribution of *Entamoeba* in humans, as determined by molecular methods.

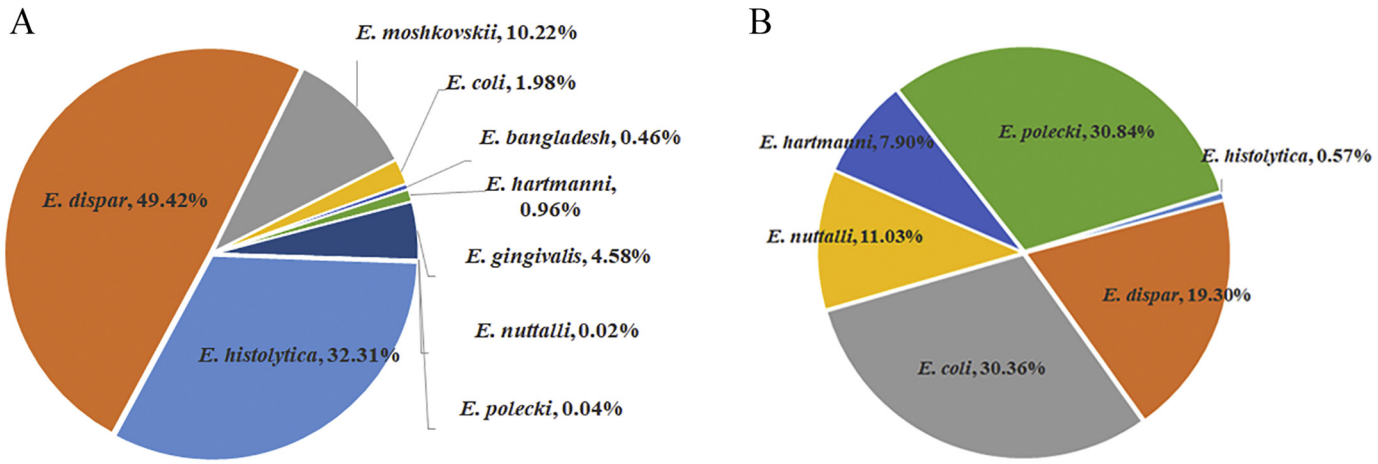

Fig. 2. Distributions of *Entamoeba* species in humans and non-human primates. A) *Entamoeba* species in humans; B) *Entamoeba* species in non-human primates.

described in NHPs include: *E. chattoni*, *E. coli*, *E. dispar*, *E. hartmanni*, *E. nuttalli*, and *E. polecki* (Feng et al., 2019). A new *Entamoeba* clade, conditional lineage 8 (CL8), was recently identified in howler monkeys (*Alouatta* spp.) in Mexico (Villanueva-Garcia et al., 2017), and high rates of *E. coli*, *E. dispar*, and *E. polecki* infection (up to 80% of the total feces analyzed) were observed in this study (Fig. 2B).

NHPs can be experimentally infected with *E. histolytica* cysts of human origin (Abd Alla et al., 2012), and no invasive disease has resulted from such experiments. Previous studies have found diarrhea in captive and wild lemurs infected with *E. histolytica,* which indicated that these lemurs may have been suffering from symptomatic *E. histolytica* (Berrilli et al., 2011; Ragazzo et al., 2018). However, the presence of *Entamoeba* and dysentery in the same host does not necessarily imply cause and effect.

1.4. Entamoeba in animals and the environment

Entamoeba have been reported in several domesticated animal species, including pigs, cattle, sheep, goats, and horses (Al-Habsi et al., 2017; Komatsu et al., 2019; Li et al., 2018; Matsubayashi et al., 2015; Matsubayashi et al., 2018; Nolan et al., 2017; Parfrey et al., 2014; Tuda et al., 2016). However, the majority of research has been conducted on pigs. In addition, *Entamoeba* have also been found in deer (Parfrey et al., 2014), rodents (Lau et al., 2014; Parfrey et al., 2014), reptiles (Garcia et al., 2014; Parfrey et al., 2014), and Asian elephants (Parfrey et al., 2014).

Entamoeba histolytica have also been detected in a variety of water sources, including wastewater in Thailand and Germany (Ajonina et al., 2018; Ferrer et al., 2012), irrigation water in Thailand (Ferrer et al., 2012), and surface water in Iran (Hemmati et al., 2015). In Japan, *Entamoeba* cysts were detected in four environmental samples from cattle farms, including soil and drinking water, based on microscopic examinations; *Entamoeba* from one soil sample was successfully sequenced and belonged to the *E. bovis* cluster (Matsubayashi et al., 2018). The amount of *E. histolytica* detected in the environment indicates that this species represents a public health risk.

1.5. Evolution of Entamoeba

Entamoebae were once widely considered to be the most primitive extant eukaryotic group—Archezoa (Baldauf, 2008). Because of the lack of the typical eukaryotic "mitochondria" organelles, *Entamoeba* was deemed a living relic, a paradigm for the earliest eukaryotic cell (Clark, 2000). Furthermore, several morphologic studies indicated that typical Golgi and endoplasmic reticulum structures were believed to be absent from *E. histolytica* trophozoites (Ghosh et al., 1999). Based on these morphological features, some consider *E. histolytica* to be a primitive eukaryote that diverged from other eukaryotes prior to development of these organelles (Dong et al., 2004).

Recently some genes of mitochondrial origin, such as mitochondrial-type cpn60 or HSP70 and Valyl-tRNA synthetase genes, have been found in *Entamoebae* nuclear genomes (Hashimoto et al., 1998; Horner and Embley, 2001). It is therefore likely that their ancestor once possessed mitochondria that were subsequently lost. Using DNA topoisomerase II as a molecular marker to reconstruct a molecular phylogenetic tree that facilitated analysis of the evolutionary position of the four amitochondriate species (*Giardia lamblia*, *Trichomonas vaginalis*, *E. histolytica*, and *Encephalitozoon cuniculi*), it was revealed that these amitochondriate protozoa evolved after and not before the acquisition of mitochondria (He et al., 2005). The presence of a continuous ER in *E. histolytica* adds to the accumulating evidence that *E. histolytica* diverged relatively late in evolution, and that many of its unusual features are likely due to reductive evolution during adaptation to its unique ecological niche (Teixeira and Huston, 2008).

The genome sequences of several *Entamoeba* species have been determined and offer fascinating insight into the evolution of these organisms (Das and Ganguly, 2014). In the annotation of the *E. histolytica* genome, some interesting evolutionary features of the genome have been highlighted, including the significant number of genes (at least 68) that appear to have been gained by horizontal gene transfer from bacteria (Loftus et al., 2005; Lorenzi et al., 2010). The majority of transfers appear to have been ancient, as orthologs were found in both *E. histolytica* and the highly divergent *E. invadens* (Roy et al., 2006). This large uptake of bacterial genes, which in general took place relatively early in the evolutionary history of *Entamoeba*, may have functioned as a trigger for adaptive evolution (Das and Ganguly, 2014; Trasvina-Arenas et al., 2019). Additionally, unlike *Plasmodium*, which has a stable genomic organization even among distantly related species, *Entamoeba* exhibit a high degree of genomic plasticity and instability; this may be an important feature of *Entamoeba* evolution, both within and between species (Weedall and Hall, 2011).

tRNA genes seem to be exceptionally abundant in the genome of *E. histolytica* (Loftus et al., 2005). The intergenic regions of these tRNA genes are composed of short tandemly repeated sequences (STRs), and the STR regions showed a high degree of intra-specific variation in repeat number, type, and arrangement patterns between tRNA array units (Escueta-de Cadiz et al., 2010). These particular features make them very useful as population genetic markers for quantification of *Entamoeba* evolutionary divergence. tRNA-linked STR loci have also been employed as a multilocus genotyping tool for exploring the relationship between parasite genotypes and the outcome of amoebic infection (Das et al., 2014; Gilchrist et al., 2012). Some common and

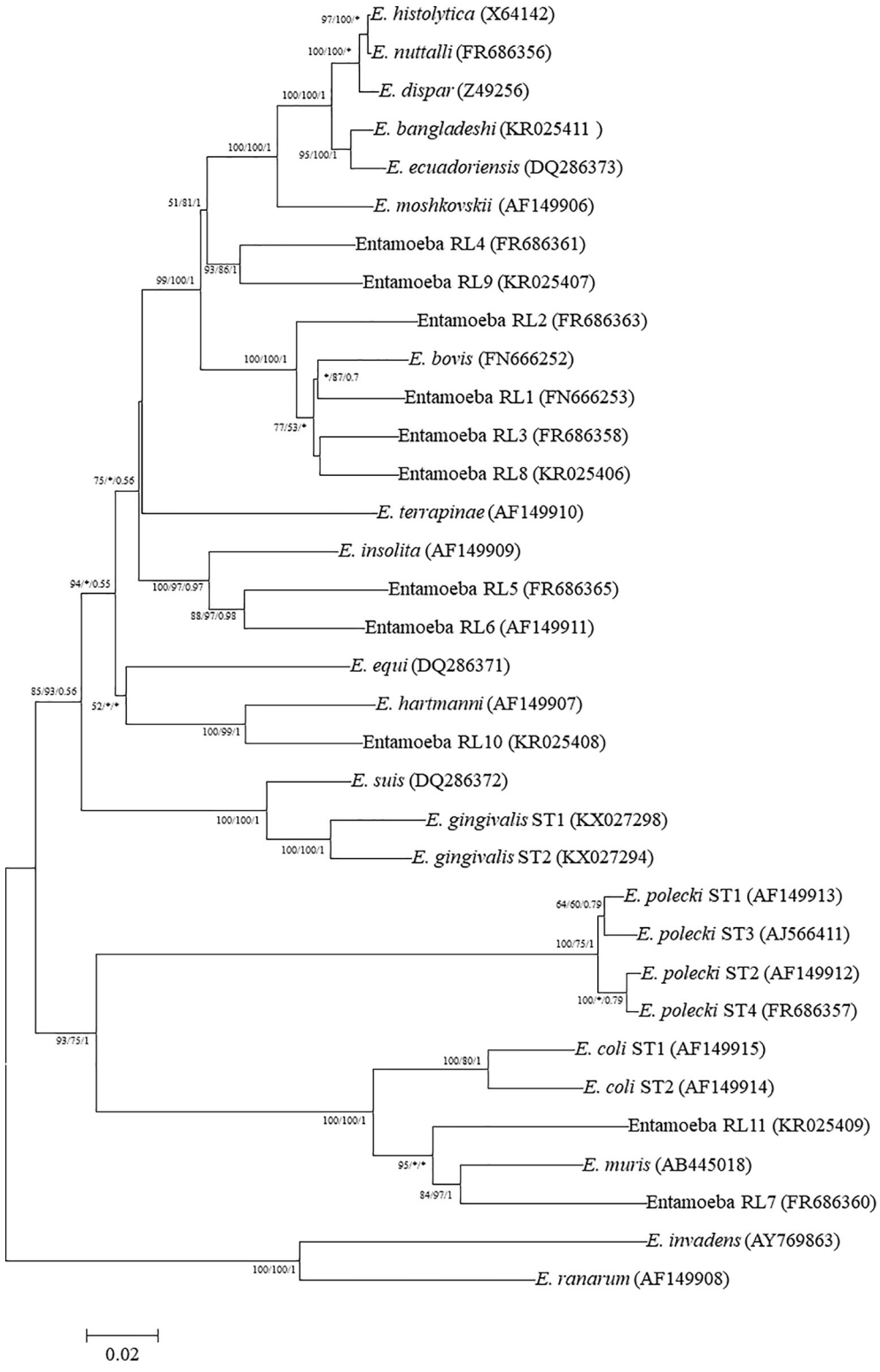

Fig. 3. Phylogenetic relationships among SSU rDNA gene sequences of *Entamoeba* species. The tree shown was inferred using the neighbor-joining method. The evolutionary distances were computed using the maximum composite likelihood method with rate variation among sites modeled using a gamma distribution (shape parameter = 0.5). The percentage of trees that clustered together based on the bootstrap test (1000 replicates) and posterior probabilities (expressed as a percentage) are shown next to the branch nodes in the order neighbor-joining/PhyML/MrBayes. An asterisk indicates a value of less than 50%; if two or three analyses produced values lower than 50%, no values are shown for that node. Accession numbers for the reference sequences are listed behind the *Entamoeba* species name.

exclusive repeat patterns that are significantly associated with disease outcome have been identified (Das et al., 2014). Moreover, the meiotic and homologous recombination-specific genes, ploidy changes, and unscheduled gene amplification of a large number of retrotransposons in the *E. histolytica* genome may result in recombination and the ability to reproduce by sexual means (Loftus et al., 2005; Singh et al., 2013; Stanley, 2005).

2. Phylogeny of *Entamoeba*

Over the past two decades, complementary developments in DNA amplification, purification, and sequencing have significantly increased our understanding of *Entamoeba* diversity (Jacob et al., 2016). *Entamoeba* SSU rDNA is a multicopy gene that is relatively fast-evolving; therefore, it provides sufficient resolution to differentiate *Entamoeba* taxa alone (Elsheikha et al., 2018). To date, numerous papers have provided insight into the *Entamoeba* phylogeny and host specificity

based on SSU rDNA analyses. In this study, a phylogenetic tree was produced using partial SSU rDNA gene sequences obtained from all currently known *Entamoeba* species in which significant ribosomal RNA gene diversity has been found (Fig. 3).

Entamoeba histolytica infection can be asymptomatic or lead to the development of severe infection with amebic colitis and amebic liver abscess in humans (Anuar et al., 2012). *Entamoeba dispar*, *E. moshkovskii*, and *E. bangladeshi* are morphologically indistinguishable from *E. histolytica* in their cyst and trophozoite forms. Phylogenetic reconstructions have consistently placed *E. histolytica* as a sister lineage to a clade that consists of *E. dispar*, *E. nuttalli*, *E. moshkovskii*, and *E. ecuadoriensis*. *Entamoeba moshkovskii* was initially thought to be a free-living protozoan species, but a recent study suggested that it causes diarrhea and colitis in infants (Shimokawa et al., 2012), whereas *E. dispar* and the newly described *E. bangladeshi* are considered non-pathogenic. *Entamoeba ecuadoriensis* has only been isolated once from sewage, and is considered to be potentially free-living (Stensvold et al., 2010a; Stensvold et al., 2011). *Entamoeba nuttalli* is prevalent in wild and captive macaques and phylogenetically closest to *E. histolytica* (Tachibana et al., 2007). Recently, an asymptomatic case of a human infected with *E. nuttalli* occurred in a caretaker of NHPs in a zoo (Levecke et al., 2015). Invasive disease has been reported in hamsters experimentally inoculated with *E. nuttalli*, but the pathogenicity of *E. nuttalli* in humans is still unknown (Guan et al., 2018; Tachibana et al., 2007). *Entamoeba gingivalis*, which colonizes the gingival pockets of human mouths, includes at least two SSU rDNA variants of *E. gingivalis* (ST1–2) (Garcia et al., 2018), and additional diversity may exist in other hosts.

Entamoeba bovis, *Entamoeba* RLs 1–3, and the newly defined *Entamoeba* RL8 formed a strongly supported monophyletic clade. It seemed incongruent that *Entamoeba* RL3, a sequence isolated from langurs, was within a clade that otherwise consisted of sequences from ruminant artiodactyls. *Entamoeba* RL10 is a newly defined lineage that is closely related to *E. hartmanni* (Jacob et al., 2016). These two sequences consistently formed a clade with very high bootstrap support in the recovered trees. The placement of *Entamoeba* RL10 as sister to *E. hartmanni* is substantial, because the latter species has not been found to have any close relatives in previous phylogenetic reconstructions (Jacob et al., 2016; Stensvold et al., 2011).

Entamoeba coli includes two major clades that have been named ST1 and ST2. Based on sequence divergence, it would be reasonable to consider *E. coli* ST1 and ST2 to be distinct species; however, other than this sequence divergence, there are no other known differences between the two subtypes to date (Elsheikha et al., 2018).

Entamoeba RL7 is most closely related to *E. muris* (Fig. 3), which produces cysts with eight nuclei. It was originally identified in a sample from a Phayre's leaf monkey (*Trachypithecus phayrei*) (Stensvold et al., 2011), but it was subsequently detected in humans in West Africa (Jacob et al., 2016). The *Entamoeba* RL11 clade is sister to *Entamoeba* RL7 and *E. muris*, but whether it forms eight-nucleated cysts is still unknown.

Entamoeba suis produces cysts with one nucleus, infects pigs and potentially gorillas, and is considered a host-restricted species (Matsubayashi et al., 2014). *Entamoeba polecki* is composed of four subtypes (ST1 to ST4), all of which have been found in humans (Stensvold et al., 2011; Verweij et al., 2001). Among these subtypes, *E. polecki* from swine, *E. chattoni* from monkeys, and the isolate from ostriches (described as *E. struthionis*) represent subtypes ST1, ST2, and ST3, respectively (Stensvold et al., 2011); ST4 appears to be the most common subtype in humans and has the widest geographical range (Stensvold et al., 2018). The potential for transmission between animal and human hosts for most of the subtypes remains unexplored.

Entamoeba invadens is the most widespread *Entamoeba* that infects reptiles (MacNeill et al., 2002; Kojimoto et al., 2001); however, other *Entamoeba* species, such as *E. terrapinae*, *E. insolita*, *E. barreti*, *E. testudines*, and *E. ranarum*, can also infect reptiles (Garcia et al., 2014). The various lineages of reptilian *Entamoeba* appear to form a limited number of clusters in the tree: *E. insolita* with *Entamoeba* RL6 and *Entamoeba* RL5, *E. invadens* was sister to *E. ranarum*, and *E. terrapinae* was clustered with *E. insolita*. *Entamoeba insolita*, *Entamoeba* RL5, and *Entamoeba* RL6 were all represented by single entries. However, there are no sequence data for *E. barreti*.

The current taxonomy of *Entamoeba* is largely based on SSU rDNA from *Entamoeba* species identified in vertebrate hosts. However, a recent study was conducted on *Entamoeba* spp. in non-vertebrate host species (three cockroach species). All of the sequences identified in the study were distinct from those reported from known *Entamoeba* species and considered novel *Entamoeba* ribosomal lineages (Kawano et al., 2017). More studies are needed to expand our understanding of *Entamoeba* genetic diversity.

3. Conclusions

This review provides an up-to-date overview of the prevalence and distribution of *Entamoeba* species. The global impact of amebic infection remains substantial. However, molecular epidemiological information on this pathogen is still scarce. More molecular studies are needed to further expand our understanding of *Entamoeba* genetic diversity. Evolutionary genomics data indicated that *Entamoeba* might engage in genetic recombination in their life cycle. Advanced whole genome sequencing of *Entamoeba* and other early branching protists may be helpful to elucidate the origin of their sexual reproduction and how these organisms exchange DNA. In addition, intra- and inter-specific genomic comparisons may help us identify genetic factors that are linked to virulence or associated with differential disease outcomes.

4. Search strategy

In the section “Molecular epidemiology of *Entamoebae*,” PubMed, Web of Science, and China National Knowledge Infrastructure were searched up to 1 April 2019 using the following search strategy: ((((epidemiology) OR prevalence) OR Molecular)) AND *Entamoeba*, without restriction on language or study type. Review articles and book chapters were also reviewed for references that fit the inclusion criteria. The list of articles from which data were extracted is provided in the Supplementary Material (Tables S1, S2 and S3). Data at the country level or district level were then mapped with prevalence estimates of *Entamoeba* in humans.

5. Phylogenetic analysis

Phylogenetic analyses were performed using a neighbor-joining analysis in MEGA 7.0 (http://www.megasoftware.net/) with the Kimura-2 parameter model, maximum likelihood in PhyML 3.0 (https://www.softpedia.com/get/Science-CAD/PhyML.shtml), and Bayesian in MrBayes 3.2.5 (https://www.softpedia.com/get/Science-CAD/MrBayes.shtml). Bayesian and maximum likelihood analysis used a General Time Reversible (GTR) model of nucleotide substitution with four categories of among-site rate variation and the proportion of invariant sites. Statistical support for distance and maximum likelihood trees was evaluated using bootstrapping (1000 replicates). Bayesian analysis used four Markov chain Monte Carlo (MCMC) strands, 1000,000 generations, with trees sampled every 100 generations. A consensus tree was produced after excluding an initial burn-in of 25% of the samples, as recommended.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.meegid.2019.104018.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Declarations of Competing Interests

The authors declare that they have no competing interests.

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