

5-2015

Phylogeny and Diversity of Entamoeba in Cockroaches, with an Emphasis on Periplaneta americana

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Phylogeny and Diversity of *Entamoeba* in Cockroaches,
with an Emphasis on *Periplaneta americana*

Phylogeny and Diversity of *Entamoeba* in Cockroaches,
with an Emphasis on *Periplaneta americana*

A thesis submitted in partial fulfillment
of the requirements for the degree of
Master of Science in Biology

by
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New College of Florida
Bachelor of Arts in Biology, 2009

May 2015
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This thesis is approved for recommendation to the Graduate Council.

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Abstract

While the parasitic Amoebozoan *Entamoeba histolytica* has been well-studied for its role in human pathogenesis, the biodiversity of invertebrate-inhabiting *Entamoeba* has scarcely been investigated. Using molecular methods, I searched for *Entamoeba* in the guts of cockroaches from four of the six Blattodean families. *Entamoeba* small-subunit rRNA genes were recovered from all eight species of cockroaches tested, five of which represent newly discovered hosts. Phylogenetic analysis of over 190 sequences revealed a novel and highly diverse clade of cockroach-inhabiting *Entamoeba*, separate from the clade predominated by vertebrate-inhabitants. These results double the known genetic diversity of *Entamoeba* and suggest that they may be widespread among cockroaches. While it would be premature to delineate new species based solely on the sequence data, work on other biologically relevant features of these *Entamoeba* variants could assist with that.

Acknowledgments

Thank you Dr. Jeff Silberman for training me in the lab; Courtney Loeschel for early data collection; Dr. Fred Spiegel, Dr. Steve Stephenson, and Dr. Ines Pinto for supporting me at the end; and my family for their support and love throughout.

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Chapter 1: Background

1.1 Abstract

Most members of the genus *Entamoeba* inhabit other organisms, and at least two species can live in anaerobic sediments. Vertebrate hosts include mammals, reptiles (including birds), fish, and amphibians. Non-vertebrate hosts include insects, annelids, and protists. Most species form cysts, which can have 8, 4, or 1 nucleus/nuclei. This has long been-and still is-used to classify and identify *Entamoeba*. The pathogenicity of some species, especially the human-inhabiting *Entamoeba histolytica*, is what generally attracts researchers to the genus. However, most *Entamoeba* are not known to be pathogenic, and only a portion of the diversity in the genus is represented in human hosts. To understand the diversity and evolution of *Entamoeba*, it is critical to study *Entamoeba* in non-human hosts. Prior to the development of molecular-based tools, *Entamoeba* were studied primarily by light microscopy, cultivation, and cross-infection experiments. Molecular-based tools allowed for improved characterization of mechanisms of pathogenesis, genetic diversity, and evolutionary relationships. This has begun to clarify the phylogeny of *Entamoeba* in vertebrates, but has not been applied to *Entamoeba* in non-vertebrates. Our understanding of the phylogenetic relationship between non-vertebrate inhabitants and vertebrate-inhabitants would be greatly improved by molecular data, which can provide a large number of characters for analysis. The aim of my research was to address the occurrence, diversity, and phylogeny of cockroach-inhabiting *Entamoeba* by taking advantage of the sensitivity and rich character sampling afforded by molecular methods. This chapter provides a context for this new information in two major ways. First, it explains the current

perception of *Entamoeba*, with an emphasis on species delineation, thus providing a basis for discussing similarities and differences. Second, it reviews cockroach biology, phylogeny, and the insect's relationships with microbes. In addition to providing a description of the *Entamoeba*'s environment and providing clues to the cockroach-*Entamoeba* relationship in real-time, this information helps assess the likelihood of coevolution and host transfer in evolutionary time. I aim to begin to fill the knowledge gap created by the lack of molecular data for *Entamoeba* in non-vertebrates. By presenting the information that surrounds this gap, this chapter will highlight the importance of the new data and the context for interpreting them.

1.2 Introduction

Entamoeba is an Amoebozoan genus consisting primarily of gut-inhabiting protists. Commensals or parasites have been found in mammals (Losch 1875), amphibians (Kudo 1922), reptiles (Sanders and Cleveland 1930), fish (Noble and Noble 1966) insects (Fantham and Porter 1911), annelids (Noller 1912 as cited by Bishop 1932), and protists (Stabler 1933) (Table 1). Free-living *Entamoeba* have been found in sewage and polluted water (Scaglia 1983; Tshalaia 1941). *Entamoeba*'s most distinctive morphological feature is its nucleus, which has a “bull's eye” pattern due to peripheral chromatin and a small, dense karyosome in the center. Although most *Entamoeba* are not pathogenic, the genus is most well-known for *Entamoeba histolytica*, which can cause colitis and liver abscess in humans (Councilman and Lafleur 1891, Kruse and Pasquale 1894 cited by Imperato 1981). The other confirmed pathogens are *Entamoeba nuttalli* in non-human primates (Tachibana et al. 2009) and *Entamoeba invadens* in reptiles (Hill and Neal 1954; Donaldson et al. 1975; Jacobson et al. 1983; MacNeill et al. 2002). *Entamoeba*

moshkovskii, found in humans and salty or polluted water, may also be pathogenic, though more studies are needed to confirm this (Ali et al. 2003; Shimokawa et al. 2012). *Entamoeba gingivalis* is nonpathogenic according to the CDC (2013), though it has been associated with periodontal disease and vaginal discharge (Linke et al. 1989; Foda and El-Malky 2012). It is unclear whether it contributes to these afflictions or simply tends to cohabit with other pathogens.

The life history of *Entamoeba* is best described for the human pathogen *E. histolytica* (Fig. 1). *Entamoeba* from humans have been studied more than those from non-human vertebrates, and *Entamoeba* from non-vertebrates have been studied the least. More knowledge of the latter will add greatly to our understanding of *Entamoeba* diversity. Distinguishing pathogenic human-inhabiting strains from non-pathogenic ones has been, and continues to be, a major area of study and discourse (Dobell and Jepps 1917; Sargeant et al. 1978; Guzman-Silva et al. 2013). Soon after the earliest descriptions of *Entamoeba* from humans (Losch 1875), amoebae and cysts with similar morphological features were reported from other organisms (Mackinnon 1914). Characteristics common among *Entamoeba* included a “bull's eye” nucleus, rounded shape with few or no blunt pseudopodia, and ability to produce cysts (with the exception of *E. gingivalis* from humans and *E. barreti* from turtles) (Kofoid and Swezy 1924; Taliaferro and Holmes 1924). These characteristics supported classification of these organisms to the same genus, while differentiating species prior to molecular studies frequently involved host, nuclei/cyst, shape, and size. Pathogenicity was also used for species differentiation, but has been limited in applicability due to the lack of observed pathogenicity for *Entamoeba* in most host species (Brumpt 1925 as cited by Diamond and Clark 1993).

Molecular-based studies have impacted our understanding of phylogeny, diversity, and species. Polymerase chain reaction (PCR)-based methods have allowed for *Entamoeba* from environmental samples to be classified as distinct lineages, even without cultivation (Stensvold et al. 2011). Species delineation among these lineages is a matter of discussion, complicated by the lack of a universal “species” definition. Many scientists who have uncovered genetic diversity by environmental sampling over the past two decades have been cautious about naming new species based solely on molecular data (Clark and Diamond 1997; Stensvold et al. 2011).

The human-inhabiting *E. histolytica*, *E. dispar*, and *E. moshkovskii* are morphologically nearly identical but pathogenetically different. Among human-inhabiting *Entamoeba*, molecular-based studies have improved the ability to distinguish between pathogenic and non-pathogenic lineages (Clark and Diamond 1991a). For a short period of time, there appeared to be a simple species distinction that reflected both molecular-based work and pathogenicity (Diamond and Clark 1993). In 1997, the World Health Organization recognized *E. dispar* as a separate, nonpathogenic species and gave diagnostic recommendations based on this distinction. However, further analysis of *E. histolytica* and *E. dispar* has shown that there are strains of varying virulence within each of these species as currently defined (Ali et al. 2007; Ximenez et al. 2010; Jaiswal et al. 2014). Like morphology, this too demonstrates the elusiveness of clear-cut differences in non-molecular characters to distinguish members of the *E. histolytica* clade from those of the *E. dispar* one.

Molecular-based phylogenies have been helpful for testing hypotheses regarding the evolution of characters (Clark and Diamond 1997; Silberman et al. 1999; Stensvold et al. 2010). Among species of *Entamoeba*, these characters have included nuclei/cyst, pathogenicity, and

choice of host.

While nuclei/cyst appeared to be consistent with early molecular-based phylogenies (Clark and Diamond 1997; Silberman et al. 1999), later studies revealed exceptions to this pattern (Clark et al. 2006; Stensvold et al. 2010; Stensvold et al. 2011), suggesting an additional gain or loss event for this character. The distribution of pathogenicity throughout the molecular-based phylogeny also suggests multiple gain or loss events for pathogenicity (Silberman et al. 1999). Host choice shows a more complex phylogenetic pattern when related to a molecular-based phylogeny, and may suggest multiple host transfer events (Silberman et al. 1999; Stensvold et al. 2011).

While *Entamoeba* from non-vertebrates and vertebrates have been described morphologically, molecular data are only available for VIE. This has led to a biased representation in molecular-based phylogenies and a biased perspective of *Entamoeba* diversity. Some of this diversity is reflected in the variety of non-vertebrate hosts, which includes insects: cockroaches (Lucas 1927; Kidder 1937; Meglitsch 1940; Hoyte 1961), bees (Fantham and Porter 1911), Japanese beetle larvae (Kowalczyk 1938), crane fly larvae (Mackinnon 1914; Ludwig 1946), and box elder bugs (Kay 1940); an annelid, the horse leech (Noller 1912; Bishop 1932); and several protists in the family Opalinidae (Stabler 1933; Chen and Stabler 1936; Stabler and Chen 1936).

Of the known non-vertebrate hosts, cockroaches in particular were chosen for my study because (1) screening multiple insect hosts in preliminary work revealed that cockroaches harbored the greatest genetic diversity of *Entamoeba* among insects tested (unpublished data, Dr. Jeffrey Silberman, University of Arkansas), (2) cockroach species vary greatly in habits that are

likely to effect microbial transmission, which may affect occurrence and phylogenetic diversity; for example, highly aggregative and coprophagic cockroaches may exhibit greater microbial transmission among conspecifics, which may increase infection rate and affect the phylogenetic diversity of *Entamoeba* in individual cockroaches, populations, and species, (3) their abundance and detritivory make them ecologically important, and improved knowledge of their gut microbiota may improve our understanding of the cockroach's ecological role, (4) some cockroaches are pests and may vector human-infecting *Entamoeba*, and more knowledge of possibly non-human-infecting *Entamoeba* in cockroaches could help distinguish between those using cockroaches solely for transport versus those that depend on cockroaches for survival, and (5) they are convenient because the high abundance and widespread distribution of some cockroach species (especially pests) allows for easy collection.

Some of the reasons above appeal to the idea that improved knowledge of cockroach-inhabiting *Entamoeba* will contribute to knowledge outside of protistology. For example, knowing more about the activities of these *Entamoeba* may improve our understanding of cockroach biology, as the impact of gut microbiota on host health is becoming increasingly clear (Dillon and Dillon 2004; Pflughoeft and Versalovic 2012; Vasquez et al. 2012). A single microbial species can significantly alter gut ecology. For example, *E. histolytica* caused significant changes in intestinal microbial diversity when it infected rabbits (He et al. 2012). Understanding the evolution of cockroach-inhabiting *Entamoeba* could improve our understanding of insect evolution. Molecular data from insects have recently supported the reclassification of termites as cockroaches (Inward et al. 2007; Roth et al. 2009). Knowledge of associated microbes could support or refute hypotheses regarding cockroach phylogeny, and

could reveal insight into relationships between host and commensal evolution.

This study was designed to provide knowledge on the diversity, phylogeny, and prevalence of *Entamoeba* in cockroaches. Genus-specific primers were designed in our lab and used to amplify *Entamoeba* SSU rDNA from the cockroach hindgut. Members of eight cockroach species across four of the six cockroach families were screened, with an emphasis on *Periplaneta americana* (American cockroach). Sequences were recovered from all eight cockroach species examined, five of which are newly discovered hosts. Phylogenetic reconstruction was used to visualize genetic diversity, relationships among cockroach-inhabiting *Entamoeba*, and relationships of cockroach-inhabiting *Entamoeba* to VIE. These data double the known genetic diversity of *Entamoeba*, suggest low (if any) genetic isolation among *Entamoeba* in *P. americana*, and contribute taxa to a clade of *Entamoeba* sister to the one predominated by VIE.

The rest of this chapter is organized in 5 parts- Prevalence, Diversity, Evolution, Cockroaches, and Taxonomy. In addition to clarifying the current state of knowledge, the first three parts illuminate various methodologies and their pitfalls, the difficulty of defining species, and gaps in knowledge. The fourth part centers around the host used for this study and provides additional focus on the gap I aim to fill. The fifth part revisits species concept. While this topic is woven throughout the first four parts, it will be important to address it again to discuss the combined influence of diversity and evolutionary history on species concept.

1.3 Prevalence

Amoebiasis has been estimated to effect 50 million people worldwide (Walsh 1986). An even greater number of people are estimated to have asymptomatic infections, mostly with *E. dispar* but some with *E. histolytica* (Stanley 2003).

One of the challenges of analyzing prevalence data is weighing the accuracy of different detection methods. Direct count and primer-specific PCR vary in sensitivity, and can yield different estimates of occurrence and abundance (Evangelopolous et al. 2001). Molecular methods are generally regarded as being the most sensitive, largely due to amplification of specific DNA by PCR (Troll et al. 1997). Use of PCR and enzyme-linked immunoabsorbent assay (ELISA) has improved detection and effected estimates of the relative abundance of *E. histolytica* and *E. dispar* (Stanley 2003). With high sensitivity comes the challenge of discerning which results are meaningful, as the DNA of organisms that are not active in that environment may also be picked up.

Prevalence of *E. thomsoni* has been found to vary among cockroach colonies. Lucas (1927) found *Entamoeba thomsoni* in all three *Blatta orientalis* (oriental cockroach) colonies she studied. High incidence of infection was reported for *P. americana* and *B. orientalis*, but specific numbers were not provided. In contrast, Hoyte (1961) found *E. thomsoni* in only a low percentage of *B. orientalis* specimens. The *Entamoeba* described as *Endamoeba philippinensis* by Kidder (1937) was found in approximately 10% of *Panesthia javanica* (additional details on *E. philippinensis* in “History of Discovery”). In future studies, better quantification of sampling and occurrence would be helpful in determining *Entamoeba* prevalence and factors that effect it.

The increased proximity among captive animals may increase infection rate. This effect

may be especially great among cockroaches, whose coprophagic behavior is ideal for fecal-oral transmission. Infection rates may also increase due to greater fecal contact due to crowding. This hypothesis is supported by Meglitsch (1940), who found that housing wild *B. orientalis* together increased protist abundance and infection rate. The method used to house cockroaches after capture could effect the perceived infection rate. These effects should be taken into account when estimating prevalence rates in the wild using laboratory data.

1.4 Diversity

Taxonomically relevant differences between organisms may be genetic, biochemical, ecological, behavioral, or morphological. Such differences among *Entamoeba* include SSU rDNA, pathogenicity, pathogenesis-related genes and proteins, diet, growth rate, host, nuclei/cyst, and social behavior. The perceived diversity of the genus *Entamoeba* has increased over the 110 years since the genus was erected (Schaudinn 1903), and continues to increase with the discovery of additional sequence diversity revealed by PCR-based methods (Stensvold et al. 2011). Many molecular biologists have been cautious about converting groups inferred from SSU rDNA alone into species definitions (Clark and Diamond 1997; Stensvold et al. 2011). These biologists require evidence in other diagnostic characters to warrant redefining species boundaries.

The diversity of NVIE has been explored relatively little compared to that of VIE, and what is known is primarily morphological and host-based. The differences among NVIE across other characters- such as those genetic, biochemical, or behavioral- are mostly unknown.

Species categorizations are ever-changing for asexual organisms, for which the biological

species concept is not applicable. The criteria used for delineation are not universally agreed upon, and new information necessitates repeated assessment of definitions.

1.4.1 History of Discovery

A brief history of pre-molecular research will provide a basis for understanding recent discoveries of diversity. Amoebiasis, characterized by bloody, mucus-filled diarrhea, had been observed for centuries before the causative agent was discovered in 1875 by Fedor Lesh, and the causal relationship supported by Fritz Schaudinn (1903) who named it *Entamoeba histolytica* (Imperato 1981). The causal link was further strengthened by reinfection of cats from cysts and feces (Darling 1913; Boeck and Drbohlav 1925; Chang 1945) and *in vitro* cultivation (Boeck and Drbohlav 1925). Not all human-inhabiting *Entamoeba* are pathogenic. Schaudinn (1903) differentiated the pathogenic *E. histolytica* from non-pathogenic *Entamoeba coli*, and Emile Brumpt differentiated *E. histolytica* from the non-pathogenic *E. dispar* (Brumpt 1925 cited by Pinilla et al. 2008). Three more non-pathogenic species-*Entamoeba hartmanni*, *Entamoeba coli*, and *Entamoeba polecki*-were later found in the gut, as well as the possibly pathogenic *Entamoeba moshkovskii*, which was originally found in sewage effluent (Casagrandi and Barbagallo 1897 cited by Imperato 1981; Clark and Diamond 1991a; Shimokawa et al. 2012; Yakoob et al. 2012). Another *Entamoeba* with unique habits is *E. gingivalis*. It can inhabit the human mouth, where it is associated with periodontal disease (Smith and Barrett 1915; Linke et al. 1989), or colonize the genital tract where it may be associated with excessive vaginal discharge (Clark and Diamond 1992; Foda and El-Malky 2012).

There was disagreement in the early 1900s regarding use of the genus names

“Endamoeba” and “Entamoeba.” *Endamoeba blattae* was described in cockroaches in 1879 (Leidy) as a novel genus and species. Many scientists maintained that the genus “Entamoeba” should never have been created, because “Endamoeba” was coined first and should therefore have had priority (Craig 1916). These scientists continued to add new species to Endamoeba, as in *Endamoeba histolytica*, *Endamoeba ranarum*, and so on. Most of these “Endamoeba” are *Entamoeba* in modern taxonomy. The only previously named *Endamoeba* that retains its generic name is *Endamoeba blattae* from cockroaches. It is distinguished from *Entamoeba thomsoni* by its larger size and large nucleus with refractive granules, thick membrane, and lack of a central karyosome (Lucas 1927; Meglitsch 1940). A few cockroach-inhabiting taxa previously placed in *Endamoeba* do not belong to the genus as currently defined. The organism originally named *Endamoeba philippinensis* is likely *Entamoeba* due to the compact centralized karyosome in its nucleus (Kidder 1937; Neal 1967). Kidder (1937) recognized the resemblance of the nucleus to that of *Entamoeba coli*, but placed the organism in *Endamoeba* due to uncertainty in the nomenclature at the time.

Like *Entamoeba* and *Endamoeba*, *Endolimax blattae* is also an amoeba that lives in cockroaches. *Endolimax blattae* can be distinguished from *Entamoeba blattae* by its 2-3 branch-like pseudopodia, large central mass of chromatin in the nucleus, and smaller size (3 to 15 micrometers) (Lucas 1927).

Among non-vertebrates, *Entamoeba* have been reported from nine arthropods, one annelid, and six protists. Reports from each host are few, and for some are limited to the original discovery. *Entamoeba apis* is the first NVIE known, discovered in bees in 1911 (Fantham and Porter) with no description provided aside from its resemblance to *Entamoeba coli* in humans.

Discovered next was *Entamoeba aulastomi* in horse leeches (Noller 1912), *Entamoeba minchini* in tipulid larvae (Mackinnon 1914), *Entamoeba belostomae* in giant water bugs (Brug 1922), *Entamoeba thomsoni* in cockroaches (Lucas 1927), and *Entamoeba polypodia* in box elder bugs (Kay 1940).

Though most NVIE are from animals, Stabler and Chen (1936) reported *Entamoeba* (which they called Endamoeba) from opalinids in frog rectums (Stabler 1933; Chen and Stabler 1936). No *Entamoeba* have been reported from protists since then. More commonly, *Entamoeba ranarum* is found extracellularly in the frog intestine. While the proximity could make it appear that the *Entamoeba* in opalinids are the same as those in the gut lumen, this is unlikely because opalinids absorb nutrients rather than phagocytose them, so opalinids would not be expected to ingest *Entamoeba* from the lumen. Also, the *Entamoeba* in opalinids had one nucleus/cyst, distinguishing them from four nuclei/cyst *E. ranarum*.

1.4.2 Morphological Differences between Genera

Non-*Entamoeba* amoebae in humans include *Iodamoeba butschlii*, *Endolimax nana*, and *Dientamoeba fragilis*. A few of the most apparent characteristics that distinguish them from *Entamoeba* are summarized here. *Endolimax nana*, originally named *Entamoeba nana*, is 5-10 micrometers across, forms spherical or oval cysts, and has an irregularly shaped mass of central chromatin in its nucleus (Wenyon and O'conner 1917). *Iodamoeba butschlii* was provisionally named *Entamoeba butschlii* when first reported but was carefully distinguished from *Entamoeba* by Dobell (1919)(Prowazek 1912 as cited by Dobell 1919). It forms an oval or irregular cyst and has a karyosome that spans half to a third of the nucleus, surrounded by smaller granules. It also

has a large iodine-attracting vesicle, hence the name *Iodamoeba*. The trophozoites of *Dientamoeba fragilis* generally have two nuclei, each having chromatin in a large central karyosome (Jepps and Dobell 1918). They do not encyst. *Iodamoeba butschlii* and *E. nana* are generally regarded as non-pathogenic (Dobell and Jepps 1917; CDC 2013). *Dientamoeba fragilis* has been associated with symptomatic and asymptomatic infection (Jepps and Dobell 1918; Johnson et al. 2004).

Members of *Entamoeba* share some features in addition to the “bullseye” nucleus. The shape is generally round with 1-3 broad pseudopodia. A few, such as *E. gingivalis*, are non-encysting. The rest form a spherical cyst that has 1, 4, or 8 nuclei at maturity, depending on species.

1.4.3 Cultivation

Generally, cultivation can be helpful for medical diagnosis and species characterization, especially when a single "species" can be cultivated by itself, or at least without other similar species. The culture can be used for reinfection experiments, or to determine the conditions required for growth. It can also provide a high concentration of the organism for morphological study. It is helpful to look at many specimens so that various morphotypes within the "species" can be observed and recorded to aid in future identification of environmental samples.

The first in vitro cultivation of *Entamoeba* was on blood agar with a single bacterium species (Musgrave and Clegg 1904 cited by Imperato 1981). The first success with a non-host-derived medium involved Locke egg-serum (LES) (Boeck and Drbohlav 1925) and was unexpected: Stool from a missionary to Africa who was dysenteric two years earlier was

inoculated into LES to cultivate flagellates (Boeck and Drbohlav 1925). Amoebae with *E. histolytica*-like nuclei were observed in the stool. In culture, these amoebae were much more prevalent than flagellates after four days, and were maintained by serial culturing for eight months. Two media were used for *Entamoeba* cultivation, each consisting of a solid phase overlaid with liquid. The first, mentioned above, consisted of coagulated whole egg covered with Locke's solution: human serum (8:1). A second biphasic medium was also successfully used by Boeck and Drbohlav (1925), similar to LES but with blood agar instead of egg. The identity of the amoebae was confirmed by morphology and inoculation of four kittens, which resulted in amoebic colitis and dysentery, as well as hepatic abscess in one kitten. Amoebae were successfully cultivated from infected kitten feces, demonstrating the presence of a pathogenic agent and supporting its identity as *E. histolytica*. Dobell and Laidlaw (1926) further improved cultivation by comparing the results of various combinations of coagulated horse serum, coagulated egg, liquid horse serum, egg-albumin, and rice starch. Axenic cultivation of *E. histolytica* was achieved in a biphasic medium of coagulated horse serum overlaid with diluted nutrient broth, chicken embryo extract, and vitamin supplement (Diamond 1961).

A few NVIE have been cultivated in vitro using a method similar to that for *E. histolytica*. Horse serum is coagulated by heating in a slanted tube and overlaid with liquid and rice starch. *Entamoeba aulastomi* from horse leech was cultivated to high numbers in this medium (Bishop 1932). *E. thomsoni* was cultivated from *P. americana* in a 1:20 dilution of inactivated human blood serum in .5% NaCl solution (Smith and Barret 1928). The identity of this *Entamoeba* was supported by Taliaferro (1928) and observations of his prepared slides by Catherine T. Lucas, the original species descriptor (Lucas 1927).

1.4.4 Molecular-based Study: Ribosomal DNA

The method of riboprinting was developed to quickly and easily distinguish between strains of *Entamoeba*, with the aim of exploring the diversity and phylogeny of many *Entamoeba* isolates and aiding diagnostics (Clark and Diamond 1991a,b; Clark and Diamond 1997). In riboprinting, PCR-amplified SSU rDNA is digested with a restriction enzyme, and the resulting set of fragment sizes are compared among isolates to help distinguish lineages of *Entamoeba* (Clark and Diamond 1991a). The process is repeated with 11 enzymes for greater coverage of DNA, increasing the likelihood of detecting diversity. Clark and Diamond (1991a) first used riboprinting to clarify the relationship of isolates described as "*E. histolytica*-like" and *E. moshkovskii*. Five isolates of *E. histolytica*-like amoebae had identical riboprint patterns. Of the eight *E. moshkovskii* isolates, there were five distinct patterns. Distance matrices based on the riboprint data revealed that four of these patterns formed a closely related group, one of which was identical to riboprints of the *E. histolytica*-like amoebae. The fifth riboprint, EC, was unique among the five riboprints, and was more similar to the riboprint of *E. histolytica* than to that of *E. moshkovskii*. This strain was later described as a distinct species, *E. ecuadoriensis*, by the same authors (Clark and Diamond 1997). The ability of riboprinting to predict diversity is remarkable, considering that it only samples 5-15% of the SSU rDNA amplicon (Clark 1997; Clark and Diamond 1997). Additional isolates from water, humans, non-human primates, pigs, reptiles, and frogs were also assessed by riboprinting (Clark and Diamond 1997). The findings include: high intra-species diversity among *Entamoeba coli* isolates; two distinct *E. gingivalis* strains, which agree with previous evidence of different isoenzyme patterns between the two

isolates (C. C. Cunnick, CGC, and LSP, unpublished data cited by Clark and Diamond 1997); seven isolates of *E. invadens* from turtles, a lizard, and snakes that had identical riboprints; and a lack of intra-specific variation among *E. histolytica* and *E. dispar* isolates. Studies of tRNA associated genes have since revealed greater diversity within *E. histolytica* and *E. dispar*. Some, but not all, of these studies have supported a relationship between genotype and virulence (Ali et al. 2007; Ximenez et al. 2010; Zermeno et al. 2013). This shows one of the limits of the resolution of riboprinting for distinguishing virulent strains from non-virulent. Though riboprinting can distinguish between *E. histolytica* and *E. dispar*, it may be unable to distinguish between virulent and avirulent strains within each of these species.

1.4.5 Molecular-based Study: Pathogenicity

Only three *Entamoeba* species are confirmed pathogens, so pathogenicity is not a useful phylogenetic character for most *Entamoeba*. However, the distinction between pathogenic and non-pathogenic *Entamoeba* has been an area of great study due to its medical relevance. If *E. histolytica* is pathogenic and *E. dispar* and *E. moshkovskii* are not, then differentiating these species would be important for avoiding unnecessary treatment. If a single species contains pathogenic and non-pathogenic strains, as has been suggested for *E. histolytica* and *E. dispar*, then distinction at a more specific level (intra-species) may be required (Ali et al. 2007; Ximenez et al. 2010; Guzman-Silva et al. 2013; Jaiswal et al. 2014).

The first molecular-based study of *Entamoeba* was an isoenzyme analysis that revealed proteomic differences between pathogenic and non-pathogenic cultures of what were called *E. histolytica* at that time, but later divided into *E. histolytica* and *E. dispar* (Sargeant et al. 1978).

Research has revealed gene expression in *E. histolytica* that differs from that in *E. dispar* and may confer virulence to the former. Some of these genes encode amoebapores for host cell lysis and oxygen-reducing proteins that may aid extra-intestinal infection (Nickel et al. 1999; Macfarlane and Singh 2006). Pathogenicity may even vary among strains of *E. histolytica*. In a study of 111 isolates from individuals in Bangladesh, short tandem repeat (STR)-containing loci were amplified from 85 isolates (Ali et al. 2007). These STR regions are highly polymorphic, potentially allowing for finer distinction of lineages. Analysis revealed a nonrandom distribution of *Entamoeba* genotypes from symptomatic and asymptomatic hosts, suggesting a link between genotype and virulence (Ali et al. 2007). Analysis of additional samples and loci also supports the existence of such distinctions among *E. histolytica* strains. Jaiswal et al. (2014) found that allelic variations in tRNA STRs correlated with clinical outcomes, including a difference between *E. histolytica* from dysenteric stool and liver abscesses. If only SSU rDNA were used, these strains would appear identical or nearly so, hence their designation as a single species. Transposable genes may add further to the complex nature of pathogenicity. The retrotransposon ehapt2 (*E. histolytica* abundant polyadenylated transcript 2) is found in *E. histolytica* but not in *E. dispar* (Willhoeft et al. 2002). Occasional retrotransposition of ehapt2 may alter gene expression to make *E. histolytica* pathogenic (Willhoeft et al. 2002). This could account for the variable pathogenicity of *E. histolytica*, or for pathogenesis that arises late after infection (Willhoeft et al. 2002).

The commonly cited statistic that "only 1 in 10 *E. histolytica* cases are symptomatic" probably requires revision (Walsh 1986). Clark (2000) explained that *E. dispar* is more common than *E. histolytica*, and that the statistic was based on microscopy and therefore missed the

distinction between the two species. However, Ali et al. (2007) and Jaiswal et al. (2014) found correlation between genotype and symptoms from *E. histolytica*, suggesting that virulence may vary within the lineage currently defined as *E. histolytica*. In another study comparing *E. histolytica/dispar* strains, hamsters were intrahepatically inoculated with one of three *Entamoeba* isolates, and liver damage was assessed. (Guzman-Silva et al. 2013). From most virulent to least, the isolates were *E. histolytica* EGG, *E. histolytica* HM1:1MSS, and 03C *E. dispar* (Guzman-Silva et al. 2013). These studies suggest intra-species diversity that has implications for *Entamoeba* and its effects on its host. It also highlights a limit of SSU rDNA regarding the resolution it provides for estimating diversity.

The cohabitation of some *Entamoeba* species may suggest secondary infection preceded by *E. histolytica* (Haque et al. 1998; Mukhopadhyay et al. 2002). This may be especially true in cases where *E. dispar* is found in liver abscesses, as *E. dispar* has reduced expression of several genes thought to aid in extra-intestinal infection (Nickel et al. 1999; Macfarlane and Singh 2006). An initial infection by *E. histolytica* might aid subsequent infection by other *Entamoeba*. Some strains of *E. dispar* may be virulent even without the aid of *E. histolytica*. The *E. dispar* strain ICB-ADO was isolated from a symptomatic patient and induced lesions when inoculated into the liver of lab animals. (Costa et al. 2006). Five tRNA-gene linked STRs of *E. dispar* from patient livers matched those of ICB-ADO, supporting the notion that virulence is genetically based and varies among strains.

To study the pathogenicity of *E. moshkovskii*, Shimokawa et al. (2012) injected *E. moshkovskii* Laredo and *E. dispar* into the caecae of mice. They used PCR to detect *E. moshkovskii* and determine infection status. *E. dispar* did not infect any mice, while *E.*

moshkovskii infected 71% (51/72) of mice. Of the infected mice, diarrhea was observed in 39% and dysentery in 6%. The same strains of mice that were resistant to *E. histolytica* were resistant to *E. moshkovskii*, while those more susceptible to *E. histolytica* were also more susceptible to *E. moshkovskii*. This suggests similar virulence mechanisms in *E. histolytica* and *E. moshkovskii*.

Shimokawa et al. (2012) used the same detection method as for the mice to study *E. moshkovskii* and diarrhea in infants in a Bangladesh urban slum. One thousand four hundred twenty-six diarrheal episodes occurred in the first 12 months after birth in 385 children. Four and sixty-three hundredths percent of episodes were positive for *E. histolytica*, 2.95% for *E. moshkovskii*, and .35% for *E. dispar*. At one and two months before diarrhea, most infants were negative for *E. moshkovskii*, demonstrating a temporal link between infection and symptoms. The diarrhea linked to *E. moshkovskii* was the same as that linked to *E. histolytica* in severity and duration. While this appears to suggest pathogenicity for *E. moshkovskii*, only two of the 42 *E. moshkovskii* samples lacked other enteropathogens, so it is possible that *E. moshkovskii* was not the lone causative agent.

Entamoeba moshkovskii appears to have broad habits and a broad habitat range. It has been reported in humans and salty or polluted water (Clark and Diamond 1991a; Tshalaia 1941; Scaglia 1983), and may or may not be pathogenic (Ali et al. 2003). There are at least six genetic variants of *E. moshkovskii* based on SSU rDNA (Clark and Diamond 1997). Many of the *E. moshkovskii* reported from humans are genetically similar, suggesting that some variants may be more likely to be non-free-living. For most isolates and habitats, clear relationships between strain and habitat remain to be demonstrated.

Similarities in the life cycle of and symptoms from *E. invadens* and *E. histolytica* lead to

the prediction of similarities in molecular expression too. Study of the *E. invadens* genome has revealed multiple homologs to the gene domains in *E. histolytica* for lectins, for attachment; cysteine proteases, for breakdown of host extracellular matrix; and amoebapores, for host cell lysis (Wang et al. 2003). For *E. invadens*, pathogenicity has been associated with host identity, but not with *Entamoeba* strain (Meerovitch 1958b). Riboprinting of seven *E. invadens* isolates showed no variation in pattern, so intraspecific genetic diversity may be relatively low or relatively cryptic (Clark and Diamond 1997). Snakes inhabited by *Entamoeba* generally show intestinal ulceration, while turtles and tortoises with *Entamoeba* have been both asymptomatic and symptomatic (Hill and Neal 1954; Jacobson 1983; MacNeill et al. 2002). Other reptile-inhabiting *Entamoeba*, such as *E. barreti*, *E. terrapinae*, and *E. insolita*, are not known to be pathogenic (Neal 1967).

1.4.6 Sex

While karyogamy has never been observed for *Entamoeba*, some molecular studies support the possibility of genetic exchange. Sargeant (1985) combined clonal cultures of distinct and consistent isoenzyme patterns to see if they would result in amoebae with a new pattern. To ensure that the initial cultures were clonal, each medium was inoculated with only a single amoeba. Forty-eight hours after mixing, a third zymodeme (a group sharing the same isoenzyme pattern) was found in one of 152 attempts at clonal culture. While the author recognizes the possibility of mutation, he considers it unlikely because a mutation in isoenzyme pattern had not been observed in cultures even nine years after isolation. In a later experiment, cultures were mixed in a syringe and injected into rats (Sargeant et al. 1988). Combinations of

three strains produced two hybrid patterns after 72 hours. Blanc et al. (1989) combined different zymodemes in pairs and subcultured each mixture every 24, 48, or 72 h. Three combinations produced single-cell clones with unique zymodemes, suggesting genetic exchange. When some of these were mixed again with a parent strain, the unique zymodeme predominated, showing stability of the new strain. Still, the validity of these studies is controversial. Clark and Diamond (1993) were unable to observe conversions. They also demonstrated that only a slight contamination event could result in data that would erroneously suggest conversion.

Ximenez et al. (2010) suggested that the high genetic diversity among *E. dispar* isolates and their ability to co-infect with *E. histolytica* could support the hypothesis of recombination between *E. histolytica* and *E. dispar*, and possibly the acquisition of virulence genes by *E. dispar* from *E. histolytica*. Haplotype diversity and distribution of two STR-linked tRNA loci for isolates from around the world suggest recombination (Zermeno et al. 2012). The occurrence of meiosis-related genes in the *E. histolytica* genome provides additional support for this possibility (Ramesh et al. 2005; Stanley 2005).

1.4.7 Social Behavior

While few studies have explicitly focused on communication between *Entamoeba*, one recent study on the topic demonstrated kin recognition among strains of *E. invadens*. Espinosa and Paz-Y-Mino-C (2012) fluorescently labeled one strain of *E. invadens* red and the other green. They then combined the strains in a plate and observed the mixture after 12-36 hours. Amoebae of the same color clustered together, suggesting that they recognized members of their own strain and aggregated preferentially. As a control, they combined red and green members of

the same strain. Clustering of same colored amoebae was not observed, confirming that clustering in the mixed-strain experiment was due to social behavior and not rapid reproduction.

1.5 Evolution

How do these *Entamoeba* lineages relate to one another? How did this diversity arise? Specifically, what are the patterns of gain and loss of characters over time? These are some of the questions that can be addressed by reconstructing phylogeny. Creating such a map also provides a basis for classifying organisms, one that is based on an objective reality of patterns over time. Phylogeny provides a way to articulate the relationships between extant taxa and to probe the gain and loss of characters in ancestors.

1.5.2 Phylogeny of *Entamoeba*

1.5.2.1 Monophyly of *Entamoeba*

To study the relationship of *Entamoeba* to other eukaryotes, Silberman et al. (1999) amplified, sequenced, and phylogenetically analyzed SSU rDNA of *Entamoeba* from primates, reptiles, amphibians, sewage, and a pig. A maximum-likelihood tree was constructed for these taxa and other eukaryotes, including the closely related *Endolimax nana* and *Mastigamoeba balamuthi*. The tree supported *Entamoeba* as a monophyletic group with maximum bootstrap support. Ptackova et al. (2013) also recovered strong bootstrap support for *Entamoeba* in a maximum-likelihood tree that included SSU rDNA of non-Archamoeba outgroups and the closely related genera *Pelomyxa*, *Rhizomastix*, *Mastigamoeba*, and *Iodamoeba*. Lahr et al. (2011) constructed a maximum-likelihood tree of 139 amoeboid lineages using SSU rDNA and

actin gene, and recovered strong support for the monophyly of the three *Entamoeba* taxa included.

1.5.2.2 Position in Amoebozoa and Archamoeba

Cavalier-Smith (1983) suggested that amitochondriate amoebae such as *Entamoeba* and *Pelomyxa palustris* be placed in a group called Archamoeba, the prefix “Arche” implying a primitive, amitochondriate state. He was correct in that molecular-based studies support the group’s monophyly (Milyutina et al. 2001; Fahrni et al. 2003; Stensvold et al. 2012). However, it may be less primitive than he predicted. The group's LCA was more likely to have undergone secondary mitochondrial loss than to have diverged before mitochondria were acquired by the ancestral eukaryote. Archamoeba lack canonical mitochondria and were once thought to be among the earliest branching eukaryotes, a conclusion supported by SSU rDNA analysis (Cavalier-Smith 1983). However, multi-gene analysis and the discovery of mitochondrial-derived genes and organelles later suggested a secondary loss of mitochondria and less primitive phylogenetic position (Tovar et al. 1999; Leon-Avila and Tovar 2004; Gill et al. 2007).

Multiple scientists recovered Archamoeba as a monophyletic group in molecular-based analyses. Lahr et al. (2011) recovered the group in SSU rDNA and actin gene maximum-likelihood trees, albeit with low support. Silberman et al. (1999) recovered the grouping of *Mastigamoeba* with *Entamoeba* in maximum-likelihood and parsimony SSU rDNA trees with high and moderate support, respectively. Ptackova et al. (2013) recovered Archamoeba with moderate support using SSU rDNA and a rich taxon sampling of non-Archamoeba and *Entamoeba*, *Mastigamoeba*, *Pelomyxa*, *Endolimax*, and *Rhizomastix*.

Stensvold et al. (2012) recovered monophyly of Archamoeba when they analyzed rDNA of 16 Archamoeba and 12 other Amoebozoa. Analyzing a broad taxon sampling of eukaryote SSU rDNA, Milyutina et al. (2001) recovered strong support for Archamoeba as a monophyletic group including *Entamoeba*, *Endolimax*, *Mastigamoeba*, and *Pelomyxa*. Fahrni et al. (2003) broadly sampled SSU rDNA and actin of Amoebozoa and other eukaryotes and recovered Archamoeba as a monophyletic group with moderate support in Amoebozoa.

1.5.2.3 Relationships within *Entamoeba*

Early molecular studies supported an *Entamoeba* phylogeny based on nuclei/cyst (Clark 1997; Silberman et al. 1999) (Fig. 2). However, later SSU rDNA studies revealed exceptions that refuted this hypothesis. Clark et al. (2006) found that uninucleate-encyster *E. suis* branched with non-encyster *E. gingivalis*, rather than with the other uninucleate-encysters. Later, Stensvold et al. (2010) found more evidence for paraphyly of uninucleated cyst-producers, in the form of multiple uninucleate-encysters branching within an otherwise quadrinucleate-encyster clade. Molecular data can help resolve morphological gain or loss events not apparent in phylogenies based on parsimony of morphological characters alone. Stensvold et al. (2011) analyzed a total of 91 sequences after recovering additional SSU rDNA samples from humans, non-human primates, and pigs (Fig. 3). If the difference in taxon sampling is accounted for, they recovered a branching pattern similar to that of Silberman et al. (1999).

Molecular-based phylogenies have become a popular framework for organizing taxa. Unfortunately, organisms without molecular data, such as NVIE, are excluded from such pictures. These organisms are also less likely to be included in non-phylogenetic frameworks,

such as ecological ones, simply because they are less visible to the scientific community.

1.5.2.5 Pathogenicity

Pathogenicity has not been observed for NVIE, although its absence should not be assumed. NVIE have been observed far less than VIE, making discovery of pathogenesis less likely for them. The topic of pathogenicity has also not been specifically studied for NVIE. Even among VIE, there is likely some bias in our view of the occurrence of pathogenicity. Reports of pathogenesis in vertebrates are from humans or captive primates and reptiles, as these are most likely to capture attention and encourage investigation. In reality, pathogenicity may occur for more *Entamoeba* and hosts than has been reported.

1.6 Cockroaches

This section reviews cockroach systematics, habits that may affect microbial inhabitants, and microbes associated with the host. These factors may have direct implications on the evolution and diversity of microbial inhabitants.

The phylogeny of cockroaches can be compared to that of its inhabitants to learn about their relationship over time. A close match would suggest an ancestral relationship and lack of transfer events between hosts. The habits of cockroaches may affect the transmission of microbes, which may affect the prevalence and isolation of microbial lineages. Differences in host habits may affect microbial transmission between or within host species. If isolation is high, the resulting divergence might increase the genetic diversity and host specificity of microbial inhabitants (Clopton and Gold 1996; van Hoek et al. 1998).

Reviewing the research of non-*Entamoeba* microbes in the cockroach will provide some perspective on the biotic factors of the cockroach as a habitat, the microbe-cockroach relationship in recent and evolutionary time, and the thought processes involved in discerning such relationships. Studies of cockroach-associated *Entamoeba* are reviewed here carefully, keeping in mind that in some cases, we do not know enough about these *Entamoeba* to distinguish between those merely vectored by the host and those actively inhabiting it. Improving our ability to distinguish between these will be required for interpreting *Entamoeba*'s habits in the cockroach.

1.6.1 Phylogeny and Classification of Blattodea

Cockroaches and termites comprise the monophyletic order Blattodea (Inward et al. 2007). Task specialization and unequal reproductive potential in termites are among the characters that distinguish them from cockroaches. Blattodea is diverse and speciose; about 4,500 cockroach and 2,600 termite species have been described (Gibbons et al. 1994; Kambhampati and Eggleton 2000). The six traditionally recognized cockroach families are: Blaberidae, containing many of the large tropical species; Blattidae and Blatellidae, containing domiciliary cockroaches; Cryptocercidae, consisting of wood-eaters; Polyphagidae, consisting of burrowers; and Nocticolidae, consisting of cave-dwellers (Roth 2003 as cited by Bell et al. 2007). Analysis of genes and neuropeptides supports the monophyly of all families except Blatellidae, which is paraphyletic (Inward et al. 2007; Roth et al. 2009)(Fig. 4). Termites branch among the cockroach taxa, sister to Cryptocercidae (Inward et al. 2007; Roth et al. 2009). Shared cellulase genes supports the grouping of Cryptocercidae and termites (Todaka et al.

2010). Similarity in the gut microbiota of termites and non-wood-eating cockroaches supports shared ancestry (Schauer et al. 2012).

1.6.2 Habits Likely to Effect Transmission of Gut Inhabitants

Greater microbial transmission is predicted to increase prevalence and decrease isolation of microbes. If isolation is low, host specificity may also be low. If transmission is restricted, the resulting divergence could increase genetic diversity and host specificity. Transmission is expected to occur more frequently in some host groups, such as conspecifics of an aggregative species, and less frequently between different host species, genera, or non-cohabiting populations. The following behaviors may increase microbial transmission in cockroaches: aggregation, parental care, allo- and auto-grooming, and coprophagy.

Cockroaches live primarily in tropical regions, though pest species that rely on humans for food, moisture, and warmth are found globally in human-inhabited regions. Most cockroaches are not pests, but are ecologically important detritivores in foliage, leaf litter, soil, and caves. They vary in sociality, diet, reproductive mode, parental care, and association with other animals- all habits that may effect microbial transmission and maintenance. Some cockroaches, such as *Macropanesthia rhinoceros*, are solitary. Others, such as *Cryptocercus punctulatus* (brown-hooded cockroach), live in family units. Others, such as *P. americana*, aggregate with both related and unrelated conspecifics. Some associate with other animals, which may provide opportunities for inter-species microbial transmission: *Pycnoscelus surinamensis* (Surinam cockroach) follow scent trails of ants to food and temporarily inhabit

their nests (Deleporte et al. 2002); females of *Nyctibora acaciana* lay oothecae on ant-acacia so that the ants will protect them from parasitic wasps (Deans and Roth 2003); *Nocticola termitophila* inhabit termite nests (Silvestri 1946 as cited in Bell et al. 2007); *Parcoblatta pennsylvanica* (Pennsylvania wood cockroach) may inhabit honeybee hives (unpublished observation by Dr. Allen Szalanski, University of Arkansas); and *Paratemnopteryx kookabinnensis* eat bat guano (Slaney 2001). These associations may have implications for *Entamoeba* transfer between cockroaches and other animals, especially those which are known hosts, such as bees (Fantham and Porter 1911).

Transmission of microbes by conspecific coprophagy has been demonstrated for some cockroaches, such as *P. americana* (Cruden and Markovetz 1984). Oral trophallaxis has been observed for some cockroaches, such as the wood-eating *Salganea esakii* (Shimada and Maekawa 2011). Microbes may also be transported by simply walking around. Durier and Rivault (2000) found that the intensity of this "trampling" behavior correlated with transmission of a toxic bait. It is possible that this behavior also contributes to microbial transmission. These behaviors lead one to hypothesize that many of the same *Entamoeba* variants would be found across cockroaches within a single wild population. Cockroaches kept in small cages could have even more homogeneity in the *Entamoeba* variants they harbor.

1.6.3 Vectored *Entamoeba*

In urban South Taiwan, 299 *P. americana* and 29 *B. germanica* from 11 primary schools were microscopically examined for *E. histolytica/dispar*. *E. histolytica/dispar* cysts were found on the cuticle and/or in the guts of 25.4% of *P. americana* and in the guts of 10.3% of *B.*

germanica (Pai et al. 2003). In India, 159 *B. germanica* were collected from a hospital and 120 from a residential area five km away (Fotedar et al. 1991). Cuticle and guts were used to inoculate plates and broths, which were examined microscopically with staining. *Entamoeba coli* were detected in 1.8% of cockroaches and *E. histolytica* in 0.4%. Examination of 76 *P. americana* from an Iraq hospital and residential area revealed higher infection rates than of *B. germanica* in the India study (Al-bayati et al. 2011; Fotedar et al. 1991). *Entamoeba histolytica* cysts were found on 19.7% cockroaches and in the guts of 11.8%, and *Entamoeba coli* on 47.3% and in the guts of 26.3%. In Ethiopia, body washes and gut contents of 6,480 cockroaches from four species were microscopically examined for cysts (Kinfu and Erko 2008). *Entamoeba coli* was found on the cuticle and in the gut of *B. germanica* and *Periplaneta brunnea*, in the gut of *P. surinamensis*, and was absent in *Supella longipalpa*. *Entamoeba histolytica* was found on the cuticle and in the gut of *B. germanica* and *P. brunnea*, but was absent in the other cockroach species. Among 920 cockroaches (primarily *P. americana*) from open-air shopping markets in Thailand, *Entamoeba histolytica/dispar* cysts were found in 4.6% of cockroaches and *Entamoeba coli* in 4.0% (Chamavit et al. 2011).

In these microscopy-based studies, it is possible that some true-inhabiting (rather than passively vectored) *Entamoeba* were misidentified as human-inhabiting *Entamoeba*. Scientists searching for human pathogens in cockroaches may be more likely to assign cysts to human-inhabiting species than to *E. thomsoni* or a new species. Improved characterization of cockroach-inhabiting *Entamoeba* would improve our ability to distinguish them from human-inhabiting species passively carried by the cockroach, improving our estimation of the cockroach's role in vectoring human-inhabiting *Entamoeba*.

1.6.4 Non-*Entamoeba* Vectored Microbes

Human-infecting eukaryotes vectored by cockroaches include the amoeba *Endolimax nana* (Fotedar et al. 1991); the yeast *Candida* sp. (Fotedar et al. 1991); and the worms *Enterobius vermiculus*, *Trichuris trichiura*, *Taenia* spp., and *Ascaris lumbricoides* (Kinfu and Erko 2008). Human-infecting prokaryotes include *Escherichia coli*, *Klebsiella* spp., *Pseudomonas aeruginosa*, and *Staphylococcus aureus* (Fotedar et al. 1991; Pechal et al. 2007).

1.7 Taxonomy

Newly discovered diversity and improved characterization of previously observed *Entamoeba* requires us to continually assess our classification system. *Entamoeba* can be unambiguously distinguished from other genera by its “bull's eye” nucleus. Species characterization, on the other hand, is a more complex subject. It is particularly difficult for organisms with poorly known sexual habits, such as *Entamoeba*, because they cannot be distinguished by sexual compatibility. Within *Entamoeba*, species have typically been characterized by morphology, pathogenicity, host, and genetics. It is generally agreed that a species must have the potential to create more individuals of the same species. Most would find this definition too narrow, but it is a good starting point for discussion. In common use, species are more than simply a genetic lineage, in that members share phenotypically expressed characters or behaviors that distinguish them from other species. Much of the subjectivity of species concepts lies in judging the relative importance of various characters for delineating species.

1.7.1 Identification

Species characterization requires identification, because a lineage must be distinct to be given a unique name. The host is sometimes used for identification if previous knowledge of *Entamoeba* in that host is available. For example, an eight nuclei/cyst *Entamoeba* in a patient is likely to be identified as *Entamoeba coli*, because this is the only eight nuclei/cyst *Entamoeba* known from humans. However, such a strategy is probably more useful for humans than for less studied hosts. Stensvold et al. (2010) revealed several new VIE via molecular methods and microscopy, showing that there is more *Entamoeba* diversity in vertebrates than previously thought.

Nuclei per cyst is a readily observable character that allows for some differentiation between lineages. Though there are multiple lineages with the same number of nuclei per cyst, this can still allow for the elimination of some possibilities during identification. In humans, for example, nuclei per cyst can be used to distinguish *Entamoeba coli* from *E. histolytica*, *E. dispar*, and *E. moshkovskii*. The latter three species are indistinguishable by morphology, so pathogenicity or molecular data are required. Pathogenicity is the main character responsible for their categorization as separate species. These three species are genetically similar, though not identical, so molecular data also supports the split (Clark and Diamond 1991a,b). On the other hand, strains of *Entamoeba coli* have greater genetic distance than *E. histolytica* and *E. dispar*, yet are defined as a single species; there are no differences other than genetics that consistently distinguish *Entamoeba coli* strains from each other (Clark and Diamond 1997; Stensvold et al. 2011). Genetic differences by themselves are not enough in this case to support the splitting of *Entamoeba coli* strains into multiple species.

In some cases, molecular data support the lumping of species, such as *E. polecki* and *Entamoeba struthionis* into *E. polecki* (Clark et al. 2006); in other cases, they support the splitting of a species, such as *E. histolytica* into *E. histolytica* and *E. dispar* (Clark and Diamond 1991b; Diamond and Clark 1993). Additional molecular data may soon result in additional splitting. For example, there may be pathogenic and non-pathogenic strains of *E. histolytica*, which some could argue are different species (Ali et al. 2007). There is no universally agreed upon genetic distance at which two lineages are different species. Even if there were, genetic distance by itself would be unconvincing, as a single type of evidence is usually insufficient for delineating species. Similarly, if pathogenicity was found in some *Entamoeba* isolates and not others, to convincingly argue for different species one would have to prove that they are also genetically unique. When lineages are delineated by ribosomal data and little else, scientists are often cautious about defining species. They sometimes label lineages as “ribosomal lineages” instead (Stensvold et al. 2011).

1.7.2 Host

A single *Entamoeba* species may be restricted to a single host species or genus, or it may have a broader host range. For example, *E. invadens* has been reported in lizards, snakes, and turtles (Chia et al. 2009; Hill and Neal 1954; Meerovitch 1958a; Macneill et al. 2002) *E. polecki* may be transmitted from pigs to humans (Desowitz and Barnish 1986); and *E. histolytica* can inhabit multiple primate species and experimentally cross-infect kittens (Kruse and Pasquale 1894; Samanta and Dey 2000; Verweij et al. 2003). "So in diagnostics the species of *Entamoeba* frequently is simply adjusted to the most common species found in the respective host" (Clark

and Diamond 1997 as paraphrased by Richter et al. 2008). *Entamoeba chattoni* has been reported mostly from non-human primates, but has occasionally been found in humans too. It is possible that it is transmitted to humans in situations of close and repeated contact, such as zookeeping (Sargeant et al. 1982). Morphologically identical uninucleate-encysters from various mammals have been given different names, such as "*E. bovis* in cattle, *E. ovis* in sheep, *E. suis* and *E. polecki* in pigs, *E. deblickei* in pigs and goats, and *E. chattoni* in monkeys" (Verweij et al. 2001). However, it is unclear whether these are distinct lineages or a single lineage capable of inhabiting multiple hosts (Verweij et al. 2001). Molecular data reveals diversity within the uninucleate-encyster clade, but until uninucleate-encysters from more hosts are assessed molecularly, the validity of these names will remain uncertain.

1.7.3 Pathogenicity

Many "non-pathogenic *E. histolytica*" have now been defined as *E. dispar* (Diamond and Clark 1993). It is often mentioned that symptoms only arise in 10% of people infected with *E. histolytica*, though the reason for such a high proportion of asymptomatic cases is mostly unknown (Walsh 1986). Some of the 90% that did not show symptoms are likely infected by nonpathogenic strains of *E. histolytica* or *E. dispar* (Ali et al. 2007; Ximenez et al. 2010; Guzman-Silva et al. 2013; Jaiswal et al. 2014). Others may involve strains that are only symptomatic under certain conditions. For example, the virulence of *E. histolytica* in hamsters was increased when the culture was mixed with bacteria prior to inoculation (Wittner and Rosenbaum 1970). In another experiment, *E. histolytica* was intracecally inoculated in germfree guinea pigs, some of which were then fed various bacterial monocultures (Phillips and Gorstein

1966). At least some guinea pigs in each bacterially inoculated sample developed amebic lesions using an *Entamoeba* concentration that did not produce lesions in the control group. Infection rates also varied by bacterial species (Phillips and Gorstein 1966). Spector (1935) similarly found increased pathogenesis when *E. histolytica* was intrarectally injected into kittens in combination with various bacteria. In this case also, infection patterns varied by bacterial species. This emphasizes the need for a holistic approach when analyzing gut microbiota and their influence on the host. It may be useful to study microbes that are unknown to play a significant role in gut ecology or host health, as they may influence these things in ways that are yet undiscovered.

Entamoeba thomsoni was discovered in cockroaches in 1927 (Lucas) and later reported in cockroaches by Kidder (1937), Meglitsch (1940), and Hoyte (1961). In this study, I used molecular methods to screen eight cockroach species for *Entamoeba* and to assess the phylogeny of these *Entamoeba*. Many *Entamoeba* variants were detected from the three cockroach species previously known to harbor *E. thomsoni*, as well as from five cockroach species previously unknown to harbor *Entamoeba*. We do not have the data required to relate the SSU rDNA sequences to particular morphologies, so we do not know how these variants relate to *E. thomsoni*. Suggesting species delineation for the new sequences would be premature, as the only data we have are sequences and hosts. The lack of host specificity for most clusters in my phylogenetic tree makes it difficult to define groups based on host species. Replicating the prudently cautious nomenclature of other scientists for similar types of data, I recommend that

the taxa associated with the new sequences be described as "variants" or "ribosomal lineages" until more is known. Though these data are insufficient for species delineation, they provide strong evidence for high genetic diversity of *Entamoeba* in cockroaches.

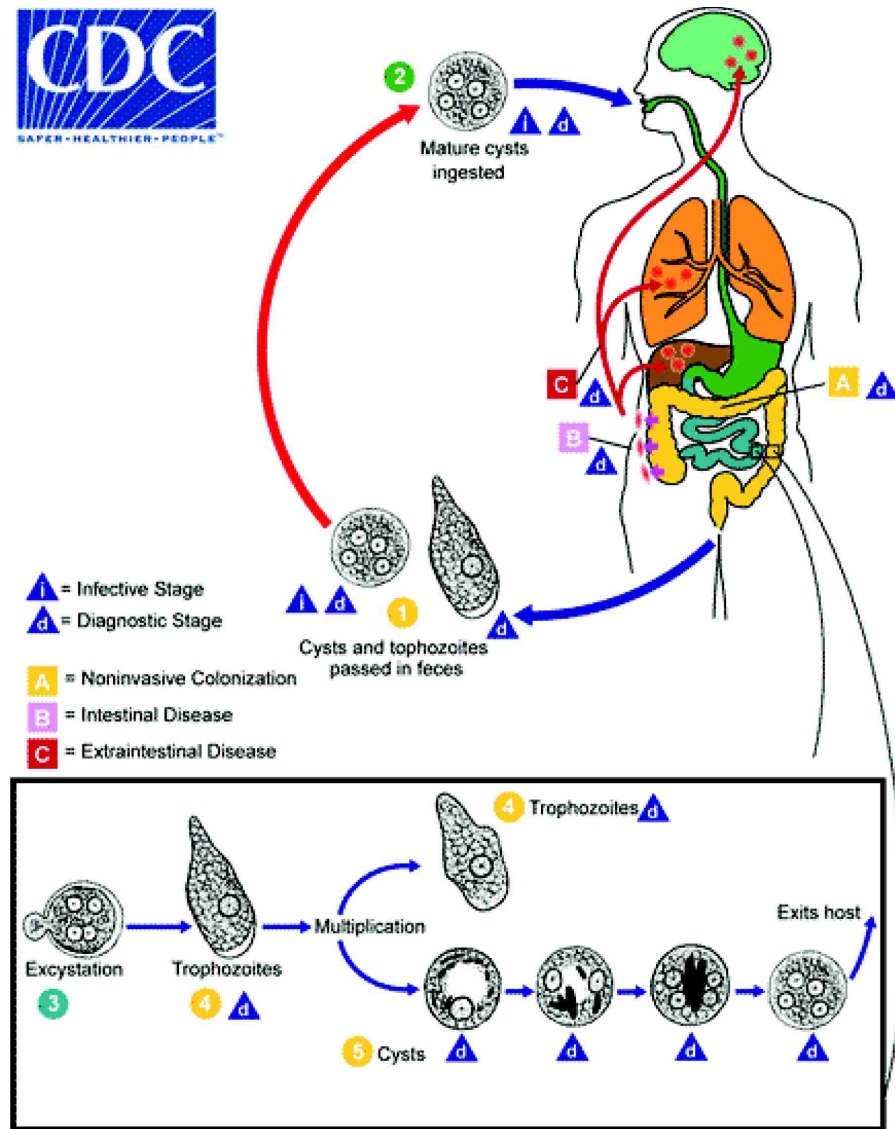


Fig. 1. From CDC (2010). “Cysts and trophozoites are passed in feces (1). Cysts are typically found in formed stool, whereas trophozoites are typically found in diarrheal stool. Infection by *Entamoeba histolytica* occurs by ingestion of mature cysts (2) in fecally contaminated food, water, or hands. Excystation (3) occurs in the small intestine and trophozoites (4) are released, which migrate to the large intestine. The trophozoites multiply by binary fission and produce cysts (5), and both stages are passed in the feces (1). Because of the protection conferred by their walls, the cysts can survive days to weeks in the external environment and are responsible for transmission. Trophozoites passed in the stool are rapidly destroyed once outside the body, and if ingested would not survive exposure to the gastric environment. In many cases, the trophozoites remain confined to the intestinal lumen (A: noninvasive infection) of individuals who are asymptomatic carriers, passing cysts in their stool. In some patients the trophozoites invade the intestinal mucosa (B: intestinal disease), or, through the bloodstream, extraintestinal sites such as the liver, brain, and lungs (C: extraintestinal disease), with resultant pathologic manifestations.”

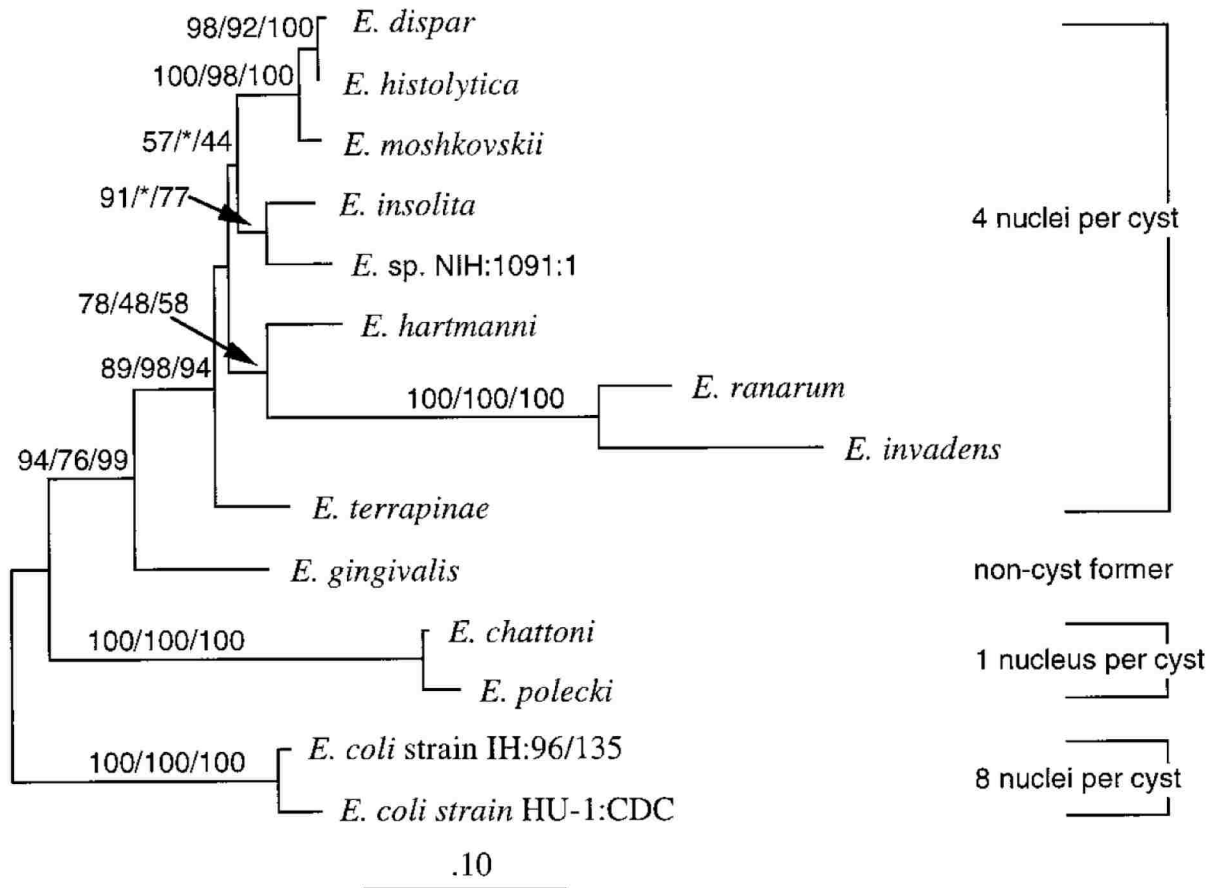


Fig. 2. From Silberman et al. (1999). "An unrooted phylogenetic reconstruction based on 16S-like rDNA exploring the relationships among *Entamoeba* species. A maximum-likelihood tree derived under a GTR model employing estimates of the proportion of invariant sites (PINVAR) and rate heterogeneity among sites (a value) is shown. Bootstrap numbers from 100 replicates of maximum likelihood, minimum evolution, and parsimony, respectively, are shown above the nodes. Nodes with significant bootstrap support are shown, and asterisks indicate bootstrap values less than 50. The scale bar represents the evolutionary distance for the number of changes per site. PINVAR 5 0.478, a 5 0.966."

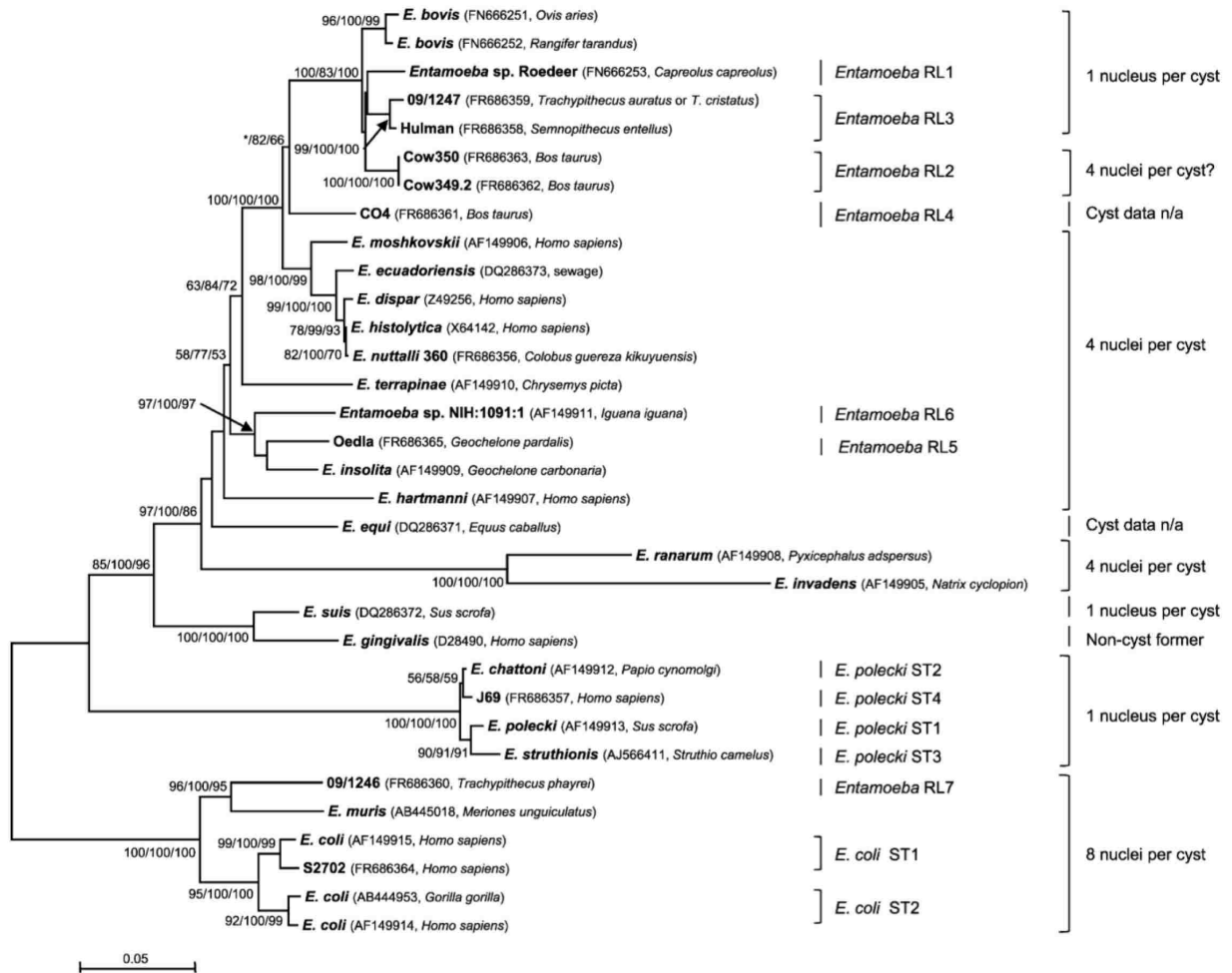


Fig. 3. From Stensvold et al. (2011). "Phylogenetic relationships among SSU rRNA gene sequences of *Entamoeba* species. The tree shown is the one inferred using the Neighbor-Joining method. The evolutionary distances were computed using the Maximum Composite Likelihood method with rate variation among sites modelled using a gamma distribution (shape parameter = 0.5). The percentage of trees clustered together in the bootstrap test (1,000 replicates) and the posterior probabilities (expressed as a percentage) are shown next to the branch nodes in the order PhyML/MrBayes/Neighbor-Joining. An asterisk indicates a value of less than 50% and if two or three analyses gave a value of lower than 50% no values are shown for that node. Accession numbers for the sequences generated in this study and reference sequences are listed parentheses with the Latin name of the host. n/a = not available. Bar = estimated number of substitutions per site."

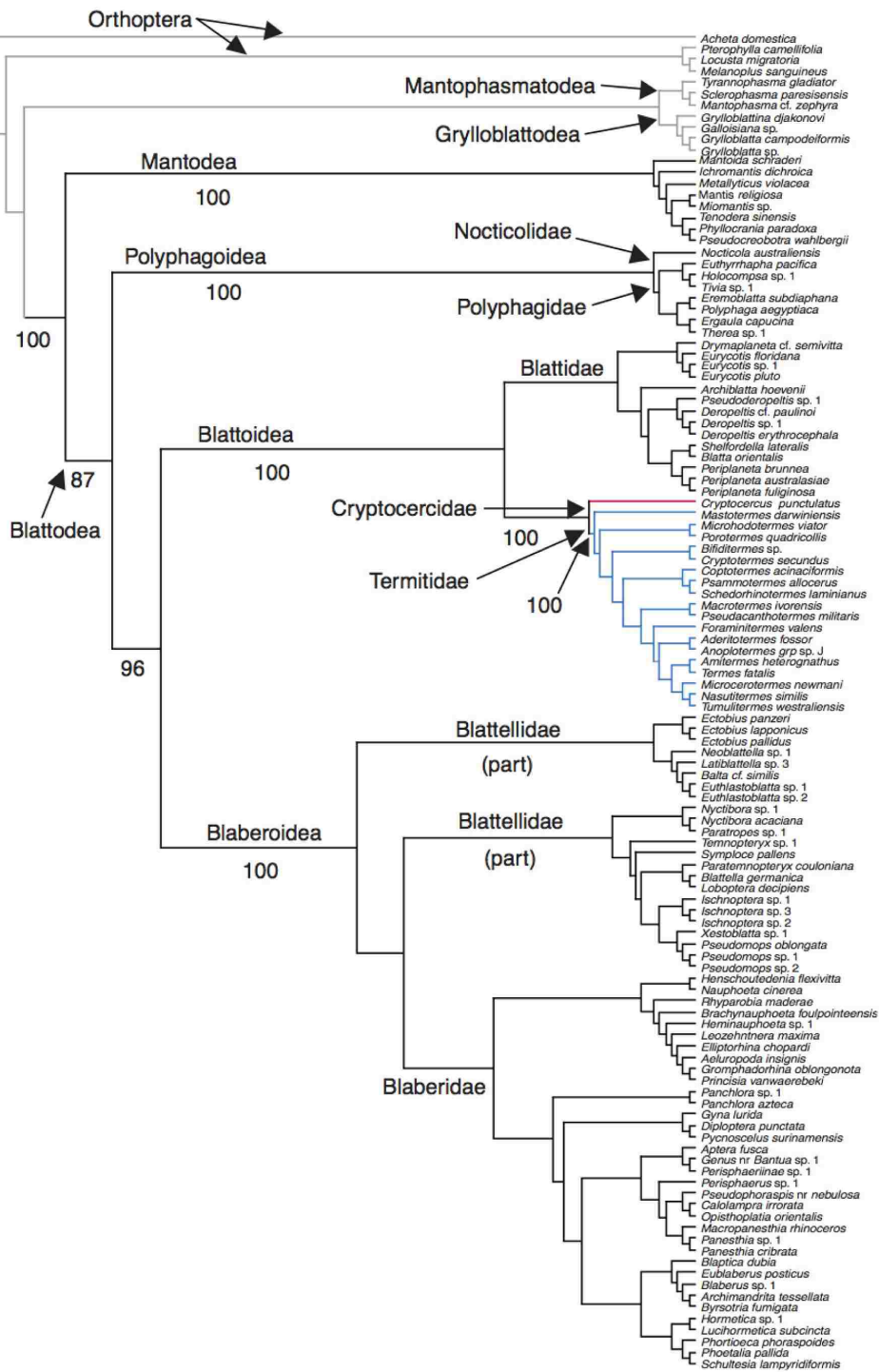


Fig. 4. From Inward et al. (2007). "Topology of Bayesian majority rules consensus tree of 2501 trees. Red branch indicates position of *Cryptocercus*, blue branches indicate termite lineage. Numbers under the branches indicate posterior probabilities (i.e. the proportion of the 2501 sampled trees that contain the node) for key nodes. Names of major clades (e.g. superfamilies) are provisional."

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Chapter 2: Analysis of *Entamoeba* in Cockroaches using SSU rDNA

2.1 Abstract

The aim of this study was to explore the evolution, diversity, and occurrence of *Entamoeba* in cockroaches by analyzing SSU rDNA. The process was enabled by primers designed in our lab that are specific for *Entamoeba* SSU rDNA. This provided us a sensitive method for detecting *Entamoeba*, as well as a method for collecting data on a large number of characters- close to 1600 for each sequence recovered. Sixty cockroaches across eight species were screened, and *Entamoeba* were detected in 56 of them. All species had at least one infected representative. Sequences were collected from 28 of these specimens. Most of the sequences were from the hindgut. Some were also recovered from non-hindgut regions, though these were predicted to be the result of contamination due to the decreased occurrence of non-gut detection as dissecting experience increased. Sequences were used to construct maximum-likelihood trees. Most new sequences formed a clade sister to the one predominated by vertebrate-inhabiting *Entamoeba*. The clade was highly diverse and composed of at least nine distinct sub-clades. The few sequences that did not belong to the novel clade grouped with *E. moshkovskii*, an *Entamoeba* previously reported in anaerobic sediments and humans, in which it is potentially pathogenic. Overall, these data have doubled the known genetic diversity of *Entamoeba* and show that infection may be widespread among cockroaches. While the genetic diversity may indicate diversity in other taxonomically significant areas, it is premature to delineate species within the novel clade until additional biological features are attributed to particular taxa or sub-clades.

2.2 Introduction

While human-infecting lineages are the most well-studied of *Entamoeba*, they represent only a small portion of the group's biodiversity. Molecular-based analyses of *Entamoeba* from humans and other vertebrates such as mice, deer, cows, and snakes revealed that *Entamoeba* is a genetically diverse genus (Clark and Diamond 1997; Silberman et al. 1999; Stensvold et al. 2011). Still, *Entamoeba* from the most populous host species have been largely ignored in phylogenetic studies. There has been little molecular data on non-vertebrate-inhabiting *Entamoeba* (NVIE) prior to this study (Chang 2010). If non-vertebrates are more abundant than vertebrates and have a high infection rate, then the most prevalent *Entamoeba* are not currently represented in phylogenetic reconstructions.

Entamoeba were often studied with regards to their pathogenicity on primates (especially humans) (Boettner et al. 2008; Tachibana et al. 2009) and reptiles (Donaldson et al. 1975; Hill and Neal 1954). However, only three *Entamoeba* species are confirmed pathogens. Studies on the pathogens are a good contribution to medical science, but to better understand the group's evolution, studying all members of the genus is crucial. Such studies would allow for mapping character-state transitions of virulence factors, in addition to other phylogenetically informative features. Both evolutionary and medical sciences would be well-served by such research.

The few reports of *Entamoeba* from invertebrates are based solely on morphology. Aside from studying the role of invertebrates in vectoring *Entamoeba*, no work has been published on *Entamoeba* in these hosts since Hoyte (1961). Little is known regarding the behavior, ecology, and phylogeny of invertebrate-infecting *Entamoeba*. Morphology by itself is insufficient for understanding the diversity of *Entamoeba* because many phylogenetically informative

differences are not superficially apparent. Nuclei per cyst is a prime example. This character was traditionally considered an important taxonomic character for identification and phylogeny of *Entamoeba*, and in early molecular-based studies it consistently correlated with groupings suggested by SSU gene analysis (Clark and Diamond 1997; Silberman et al. 1999). However, later studies revealed exceptions to this rule (Stensvold et al. 2010; Stensvold et al. 2011).

It would be phylogenetically informative to study the differences and similarities in species' habits, as it might provide a basis for distinguishing species and understanding biodiversity. However, such experiments are difficult when different species are indistinguishable by standard methods, as has often been the case for *Entamoeba* with light microscopy (Clark and Diamond 1991a; Diamond and Clark 1993; Stensvold et al. 2010). Molecular methods can help, as they can discern between lineages less ambiguously. Molecular data is especially important for species delineation within *Entamoeba* because of the paucity of morphological differences and the possibility of evolutionary convergence. Still, it is important to remember that though molecular data can help distinguish lineages, it is not generally sufficient for species delineation. Most scientists would consider molecular data with other, non-molecular aspects of the lineage before delineating species (Clark and Diamond 1997; Verweij et al. 2001; Stensvold et al. 2011).

Most *Entamoeba* form desiccation-resistant cysts that aid transmission. A cyst can have 8, 4, or 1 nucleus or nuclei. This number is consistent within a genetic lineage over a short period of time, but is not always consistent in broader phylogenetic groups (Stensvold et al. 2010; Stensvold et al. 2011). Therefore, nuclei per cyst does not necessarily indicate phylogenetic position, and is not used as the sole criterion for such positioning. At a minimum,

host identity is used in conjunction with nuclei per cyst for species characterization. Unfortunately, this can result in two names for a single genetic strain if it infects multiple host species. In such cases, molecular analysis can help delineate species. For example, *Entamoeba polecki* from pig and *Entamoeba struthionis* (Gordo et al. 2004) from ostrich were once described as separate species, but a high similarity in SSU rRNA gene sequences led Clark et al. (2006) to reclassify them as *E. polecki* subtypes rather than separate species. Conversely, molecular data can be used to distinguish between multiple *Entamoeba* lineages in the same host species, especially if they are suspected of having a different morphology or life history. For example, molecular data can be used to distinguish between morphologically identical *E. histolytica*, *E. dispar*, and *E. moshkovskii*, all of which vary in suspected pathogenicity (Clark and Diamond 1991a,b; Diamond and Clark 1993; Shimokawa et al. 2012). As these examples demonstrate, data on more than one variable are ideal for judging whether genetically distinct lineages are separate species or multiple variants of a single genetically diverse species. Data for many of these variables are missing for non-vertebrate-inhabiting *Entamoeba* (NVIE), so I will use "variant" instead of "species" for denoting the genetically defined lineages uncovered here.

Entamoeba histolytica, *E. dispar*, and *E. invadens* sometimes invade non-gut organs—most commonly, the liver (Hill and Neal 1954; Ximenez et al. 2010). NVIE have only been reported from guts, though non-gut organs of invertebrates have not been sufficiently screened. They have certainly not been screened for *Entamoeba* using molecular methods, which may have a stronger detection sensitivity than direct count by microscopy. In this study, non-gut organs of some cockroach specimens were screened for *Entamoeba* to see if migration to non-gut organs occurs in cockroaches.

Microbes are thought to have played a major role in the evolutionary trajectory and success of insects in the monophyletic order Blattodea (Nalepa et al. 2001). *Periplaneta americana* is a gregarious and abundant Blattodean that often lives in close association with humans. Such a lifestyle is likely to provide many opportunities for *Entamoeba* to travel between conspecifics or between cockroach and human. Determining the genetic lineages present in cockroaches will make it easier to study transmission, both on the time scale of a single genetic lineage as well as that of evolutionary significant transfer events. Studying *Entamoeba* phylogeny in the context of *P. americana*'s evolutionary history will improve our understanding of phylogeny in both groups and the relationship between host and microbe over time.

Prior to this study, it was difficult to estimate the contribution of NVIE to total *Entamoeba* diversity because there was no molecular data for them. We have used genus-specific primers to gather *Entamoeba* SSU rDNA sequences from eight cockroach species.

2.3 Methods

2.3.1 Roach Collection and Dissection

Of the eight cockroach species studied, *P. americana* was the most heavily sampled (Tables 1 and 2). Sixteen *P. americana* cockroaches were obtained from Carolina Biological Supply Company (Burlington, North Carolina) and 18 from the University of Arkansas, Fayetteville campus. Cockroaches from Carolina were processed at various times, some as late as 18 months after purchase. They were housed at room temperature in 26x15x15 cm plastic

cages containing 5-20 adult cockroaches and a variable number of sub-adults. They were fed dry cat food, oatmeal, and occasional fruit (approximately once a week to once a month). Wild cockroaches were hand-caught within or in close proximity to buildings. Some were temporarily housed together, while others were housed individually for up to 24 hours (*P. americana* 10 through 31) to collect specimen-specific feces. They were given oatmeal and water as needed (approximately once a week). Each cockroach was prepared for evisceration by carbon dioxide or ethyl acetate-induced knockout, followed by severance of the head. For both methods, the cockroach was first placed in a 50 mL plastic tube. For the carbon dioxide method, a tube connected to a gas tank was inserted between the partially-open lid and 50 mL plastic tube, and gas was released until the cockroach was still. Within a few minutes of knockout, the head was severed by cutting the neck with an ethanol-wiped razor blade. With this method, the legs, mouthparts, and antennae would often begin moving after a few minutes, even after decapitation. For the ethyl acetate method, a Kimwipe (Kimberly-Clark, Irving, Texas) was crumpled and inserted into the 50 mL tube after the cockroach, and 3-6 drops of ethyl acetate was added to the Kimwipe. The tube was then sealed, and reopened in a few minutes after the cockroach stopped moving. I switched from the carbon dioxide method to the ethyl acetate method because the latter appeared to cause complete cessation of movement. This method seemed more humane and also facilitated dissection because I no longer had to deal with moving parts. After decapitation, the body was pinned ventral side up on a silicone-filled dish. Iris scissors (1 cm cutting edge, Bioquip, Rancho Dominguez, California) and forceps (Bioquip) were used to remove abdominal sternites and fat to expose the digestive organs, which were then carefully excised using forceps and scissors. Dissection tools were periodically flame-sterilized to avoid

contamination. Hindgut, defined as the colon and rectum, was stored for each specimen separately at -80°C.

Specimens of the other seven cockroach species were obtained from various sources in living condition unless otherwise noted and eviscerated in the same manner as *P. americana*. Five living *Gromphadorhina portentosa* were obtained from a colony maintained by Dr. Donald Steinkraus (University of Arkansas, Entomology Department). Four *Blattella germanica* were obtained from apartments in Fayetteville, Arkansas. Three *Blatta orientalis* were obtained in close proximity to each other and to a building on the University of Arkansas campus. Four *Blaberus giganteus* were obtained from Carolina Biological Supply Company. Three *Parcoblatta pennsylvanica* were found in honeybee traps at the University of Arkansas Research Farm in Fayetteville set by Amber Tripodi (University of Arkansas, Entomology Department). The other two were collected from inside houses in Fayetteville. Three *Cryptocercus punctulatus* were collected from forest in Gatlinburg, Tennessee, and mailed to the lab two days after collection. They arrived in living condition and were frozen at -80°C until dissection. One *Periplaneta fuliginosa* was collected from inside a Fayetteville house near a doorway. It likely entered the house close to the time of capture and was not a long-term inhabitant of the house.

2.3.2 DNA extraction and Amplification

Genomic DNA was isolated from frozen organs using ZR soil microbe DNA isolation kit (Zymo Research, Irvine, California), PowerSoil Tissue & Cell Kit (MO BIO, Carlsbad, California), or UltraClean Tissue & Cells DNA Isolation Kit (MO BIO) according to manufacturer's protocol and stored at -20°C. The isolate was likely a mixture of DNA from

cockroach tissue, ingested food, and various microbial inhabitants. Small-subunit ribosomal RNA DNA (SSU rDNA) of *Entamoeba* was amplified using two consecutive polymerase chain reactions (PCRs). The first reaction was a mixture of 1.5 µl isolated DNA, 10 µl GoTaq Green Master Mix (Promega, Fitchburg, Wisconsin), 2 µl each of eukaryotic SSU rDNA primers 5'F and 3'R (10 ng/µL, 5'-AACCTGGTTGATCCTGCCAGT-3', 5'-TGATCCTTCTGCAGGTTACCTAC-3', Medlin et al. 1988), and 6 µl sterile, double-distilled water (ddH₂O). Reaction conditions in the thermal cycler (Tgradient, Biometra, Gottingen, Germany) were 94°C 30 s, 30-35 cycles of 94°C 22 s, 42°C 1 min, and 72°C 3 min, and a final step of 72°C 5 min. After thermocycling, 7-12 µl of the product was electrophoresed on a 1% agarose gel (agarose (Agarose I, Amresco, Framingham, Massachusetts), TA (4.84 g Trizma Base (Sigma-Aldrich, St. Louis, MO) and 1.14 g glacial acetic acid up to 1 L with ddH₂O) or TAE buffer (TA buffer with 0.37 g EDTA), and 0.34 µg/mL ethidium bromide) and viewed and photographed with a 302 nm transilluminator (BioDoc-It Imaging System, UVP, Upland, California) to verify amplification. *Entamoeba* SSU rDNA-specific primers, 1F and 1700 R (5'-TGGTTGATCCTGCCAGTATTA-3', 5'-CATCTTGGGCYGCACGC-3'), were designed in our lab by Dr. Jeffrey Silberman. For the second reaction, 0.5-1.0 µl of product from the first reaction was added to 10 µl GoTaq Green Master Mix (Promega), 2 µl of each of 1F and 1700R (3.5 pmol/µl), and 6 µl sterile ddH₂O. The mixture was thermocycled at 94°C 30 s, 30-35 cycles 94°C 22 s, 52°C 1 min, and 72°C 2 min, and finally 72°C 5 min. Seven to 12 µl of the reaction product was run on 1% agarose gel to visualize amplified DNA using a UV transilluminator as described above. Non-hindgut organs were retrieved from some *P. americana*, *B. germanica*, and *B. giganteus* specimens to determine whether *Entamoeba* travel outside of the hindgut

analogous to the behavior of the human pathogen *E. histolytica*. These non-hindgut organs included: ventriculus, caeca, proventriculus, crop with esophagus, malpighian tubules, fat, ovary, testis, head, leg muscle, whole leg (unwashed), cuticle (unwashed), and egg case (washed and crushed). The expected band size for *Entamoeba* using primers 1F and 1700R was 1600 base pairs (bp). All cockroaches listed as infected had positive bands. Some cockroach specimens with this band were processed further to recover sequences: Six *P. americana* from Carolina Supply, 5 *P. americana* from the wild, 1 *B. germanica*, 1 *B. giganteus*, 3 *B. orientalis*, 1 *C. punctulatus*, 5 *G. portentosa*, 5 *P. pennsylvanica*, and 1 *P. fuliginosa*. For these 28 specimens, bands of the expected product size were excised and placed in an aerosol barrier tip (Sorenson BioScience, Salt Lake City, Utah) cut to fit a 1.5 mL microfuge tube. This was spun at 20,800 xg for 5 minutes to elute the DNA + buffer solution from the agarose.

2.3.4 Separation of Variable Sequences

The gel-purified DNA potentially contained a mixture of SSU rDNA from multiple genetic variants of *Entamoeba*. These variable sequences were separated by inserting them into plasmids which were then inserted into *E. coli*.

Half a microliter, or 1 μ l for faint bands, of gel-purified DNA was ligated into pCR4-TOPO plasmids (Invitrogen, Life Technologies, Carlsbad, California) by combining the following in the order listed: sterile ddH₂O to total volume of 3 μ l, 0.5 μ l salt solution (1.2 M NaCl, .06 M MgCl₂), 0.5-1 μ l gel-purified DNA, and 0.5 μ l pCR4-TOPO (10 ng/ μ l plasmid DNA). This mixture was incubated at room temperature for 15 minutes. Twenty-five microliters of Mach1 chemically competent *Escherichia coli* (Invitrogen) was added to the

mixture, which was then gently stirred and incubated on ice for one hour. Plasmid uptake was induced by heat shock in a water bath at 42°C for 30 s, followed by placing the tube in ice for 1 min. Two-hundred fifty microliters of SOC Medium (Invitrogen) was added before the mixture was rotated on a rotisserie for one hour at 37°C. To avoid excessive liquid on the plates, cells were concentrated prior to spreading on LB/antibiotic/XGAL agar plates.

To make LB/antibiotic/XGAL agar plates, 1 L of distilled water was added to 20 g LB (Luria-Bertani Broth, Sigma-Aldrich) and 15 g agar (agar-agar, EMD Millipore, Merck KGaA, Darmstadt, Germany). The mixture was autoclaved, poured into 15 mm (depth) Petri plates, and allowed to cool overnight before storage at 4°C. In some cases, the antibiotic was added to the agar mixture when it had cooled enough to prevent denaturation of the antibiotic, but was still liquid enough for pouring. In other cases, it was spread onto the solidified agar approximately one to three hours before *E. coli* inoculation. The final concentration of kanamycin or ampicillin was always 50-100 µg/ml. XGAL was spread onto the agar approximately one to three hours before *E. coli* inoculation. Cell concentration involved centrifugation at 8000 rpm for 1 min., discarding 150 µl supernatant, and resuspending the pellet in the remaining supernatant. pCR4-TOPO plasmids contain an ampicillin and kanamycin resistance gene, so only cells that contain the plasmid are expected to survive plating. The XGAL is a sugar that is processed into blue pigment when the LacZ gene is intact. The insertion site for ligated DNA is within this gene and insertion of foreign DNA interrupts its function. Cells will appear white if foreign DNA was inserted into this site. The plates were incubated at 37°C for 12 to 16 hours and up to 30 white colonies were selected for each cockroach. Each colony was touched with a pipette tip to pick up cells, followed by dipping the tip into a mixture of 10 µl GoTaq Green Master Mix

(Promega), 2 µl of each of 1F and 1700R primers (3.5 pmol/µl), and 6 µl sterile, double-distilled water. The PCR was thermocycled at the same parameters as the nested PCR earlier. Seven to 10 µl of the completed reaction was gel electrophoresed to confirm presence of DNA in the plasmids that corresponds with the expected size of *Entamoeba* SSU rDNA.

To select colonies with unique sequences for further processing, RFLPs were assessed. For positive colonies, 10 µl of the remaining PCR reaction was digested with 0.2 µl TaqI endonuclease (10x, Promega) combined with 0.5 µl bovine serum albumin, 2 µl Buffer E (Promega) or NEBuffer 4 (New England Biolabs, Ipswich, Massachusetts), and 7.3 µl water. The mixture was incubated at 65°C for 10-16 hours. Seven to 12 µl of the product was electrophoresed on 3.5 %, Nusieve GTG: agarose (3:1 + TA or TAE buffer). *E. coli* from colonies with unique restriction fragment length polymorphism (RFLP) patterns, as well as up to 5 for duplicates of some patterns in each host, were cultured overnight in liquid LB + ampicillin (100µg/ml). In early experiments, cells were grown in 4 mL, and 2 mL of the cell mixture was concentrated to 600 µl after 12-16 hours of rotating at 37°C. In later experiments, cells were grown in 600 µl of medium with rotation or agitation and no concentration procedure. Later, it was discovered that rotation or agitation could be omitted without negatively affecting sequence recovery. Plasmids were extracted from the cultured *E. coli* using Zyppy Plasmid Miniprep Kit (Zymo Research) following manufacturer's protocol and eluted in 50 µl EB buffer (10 mM Tris-Cl, pH 8.5, Qiagen, Venlo, Netherlands).

2.3.5 Sequencing and Phylogenetic analysis

Primers were added to plasmids and sent to the University of Arkansas DNA Resource Center for Sanger sequencing by a 3130xl Genetic Analyzer (Applied Biosystems, Life Technologies). Sequencing primers T3 (5'-ATTAACCCTCACTAAAGGGA-3', Invitrogen), T7 (5'-TAATACGACTCACTATAGGG-3', Invitrogen), and 514F (5'-GGTGCCAGCAGCCCGCGGTAA-3', Dr. Jeffrey Silberman) were used for sequencing a 1.5 kb contig. Chromatograms were visually reviewed for accuracy and contigs assembled with Sequencher 4.0 (Gene Codes). Duplicates were identified by searching for 100% sequence match in Jalview (Waterhouse et al. 2009). Unique sequences were manually aligned in SeaView (Guoy et al. 2010) to account for indel-induced frameshifts of sequences relative to one another. Aligned sequences were screened for chimeras (an artifact of PCR) using the web-based program Bellerephon (Huber et al. 2004). This program constructs a distance matrix for sequence fragments on both sides of a breakpoint. It determines the influence of each sequence on dissimilarity between the two sides by removing each sequence and recalculating the dissimilarity. Sequences that have the biggest contribution to dissimilarity (quantified as a "preference score") are reported as possible chimeras. Huber-Hugenholtz correction was selected as a parameter for the distance calculations. This function is designed to increase the weight of calculations for similar homolog pairs compared to more different ones, as chimeric sequences are more likely to arise among similar amplicons. Another parameter that can be selected is window size, which determines the number of nucleotides analyzed on each side of the breakpoint. The program was run with all window sizes available: 400 bp, 300 bp, and 200 bp. Potential chimeras were reported along with preference scores and percentage identity to

parent strands. Chimeras with identity of 100 or 99 to a parent strand were regarded as highly likely chimeras, and those with lower identities (81.9 was the lowest) were regarded as possible, though less likely, chimeras.

One alignment was constructed using all unique *Entamoeba* sequences, which included those generated in our lab of NVIE and from other scientists via Genbank of VIE. This alignment was built on one given to me from Dr. Jeffrey Silberman in September 2010. Sequences were added as they were recovered in our lab or became available on Genbank. Another alignment was built on one passed on from Dr. Lora Shadwick (University of Arkansas, February 2011), which included 141 taxa of non-Amoebozoa, Amoebozoa, and Archamoebae. An additional 47 *Entamoeba*, non-*Entamoeba* Archamoeba, and non-Archamoeba Amoebozoa were added.

Among 140 sequences from *P. americana*, two groups of duplicates were detected, with two sequences in one group and four in the other. After four duplicates were removed, 136 unique sequences remained. An alignment of these 136 sequences and 40 of VIE from Genbank was screened for chimeras as described above. Thirteen *P. americana* sequences were identified as highly likely to be chimeras, and 12 additional sequences were identified as possible, though less likely, chimeras. All 25 putative chimeras were removed to avoid overestimation of diversity.

Among 53 sequences from non-*P. americana* cockroaches, no duplicates were found. To find chimeras in this subset, an alignment with all unique *Entamoeba* sequences (258 taxa) of sufficient length was tested. *Entamoeba bangladeshi* was excluded because its sequences were too short. Among taxa from non-*P. americana* cockroach hosts, 10 putative chimeras were

detected and removed from further analysis. Seven of these were from hindgut and three were from non-hindgut regions.

Maximum likelihood trees were constructed using the RaxML algorithm on CIPRES Science Gateway (Stamatakis 2014; Miller et al. 2010). Identity of long-branched taxa as *Entamoeba* was confirmed by the similarity of these taxa to other *Entamoeba* using NCBI's BLAST.

Eighty-one sequences from *P. americana* hindgut were aligned with 39 *Entamoeba* sequences from Genbank. To assess the monophyly and rooting of Archamoeba, 7 *Entamoeba* from *P. americana*, 24 VIE from Genbank, and 17 other archamoebae were aligned with 110 Amoebozoan and 30 non-Amoebozoan taxa. A tree was constructed using 184 taxa and 1,052 unambiguously aligned characters (Fig. 1, Table 3). A subset of 73 taxa was used to construct a smaller tree that still preserved the branching of *Entamoeba* (Fig. 2). This tree included 7 *Entamoeba* from *P. americana*, 7 *Entamoeba* from vertebrates, 1 *E. moshkovskii*, 11 non-*Entamoeba* Archamoeba, 37 non-Archamoeba Amoebozoa, and 10 non-Amoebozoan outgroup taxa. The basal branches of Archamoeba from the larger tree were used as the basal branches in an Archamoeba-only tree, consisting of *Entamoeba*, mastigamoebids (*Mastigameoba*, *Iodamoeba*, *Endolimax*), pelobionts, and *Rhizomastix*. This tree consisted of 45 taxa and was calculated from 1,119 unambiguously aligned characters (Fig. 3). The basal branches of *Entamoeba* in this tree were used as the basal branches in *Entamoeba*-only trees.

Non-hindgut sequences were initially excluded from the analysis because it was unclear whether they were contaminants from the hindgut or true inhabitants of non-hindgut tissue. Contamination was suspected because *Entamoeba* was detected in non-hindgut regions in early

dissections but not in later ones. My dissecting skill improved over time and likely reduced the likelihood of contamination between regions. For the purposes of the current study, even if sequences from non-hindgut regions indicate contaminants, the contaminant probably originated from the same cockroach, so can still be included to study *Entamoeba* diversity and to compare *Entamoeba* from different host specimens. The distinction between sequences from hindgut and non-hindgut regions is not necessary for analysis of phylogeny and diversity at the level of host specimen, but a distinction is made here to reduce ambiguity or confusion in interpretation of results. Future studies may or may not corroborate a hypothesis of infection in non-hindgut regions of the cockroach.

Four *Entamoeba*-only trees were constructed from taxon subsets and 1,272 unambiguously aligned characters (Figs. 4, 5, 8, 9, 10, and 11). The first of these contained 110 *P. americana* sequences and 39 *Entamoeba* sequences from Genbank. A version of this tree with a reduced selection of *P. americana* sequences is shown in Fig. 4. The basal branches of this tree were used as the basal branches for the second tree, which consisted of the 105 *P. americana* sequences that formed a clade sister to the 39 vertebrate-inhabiting *Entamoeba* sequences in the earlier tree (Fig. 5). The other five *P. americana* sequences branched near *E. moshkovskii* within the clade predominated by vertebrate-inhabiting *Entamoeba* (Fig. 11), and were excluded from this tree. The purpose of this dataset was to study patterns within *P. americana*, the most extensively sampled host species. Taxa of particular host categories were highlighted to observe patterns, such as phylogenetic distribution of Carolina Supply vs. wild cockroaches, host specimens, and male vs. female (Figs. 5, 6, and 7).

The third *Entamoeba*-only tree contained all vertebrate-inhabiting *Entamoeba* sequences, all *P. americana* sequences, and 43 *Entamoeba* sequences from the 7 non-*P. americana* species (Fig. 11). The fourth tree was similar to the third but without vertebrate-inhabiting *Entamoeba* sequences. Characters were mapped onto this tree to study phylogenetic distribution as it relates to host species, host specimen, and location in the host (Figs. 8, 9, and 10).

2.4 Results

2.4.1 Sequences Collected

Duplicate sequences (100% match) were retrieved from each of two host specimens (2 from Pa4 and 4 from Pa24), and no duplicates were retrieved across multiple host specimens. Two to 15 unique *Entamoeba* sequences were found per *P. americana* specimen (Table 2).

One sequence was common to all four monoinfected *G. portentosa* specimens.

2.4.2 Monophyly and Relationships of *Entamoeba* and Other Archamoebae

In a tree of 32 non-Amoebozoan outgroup taxa, 106 non-archamoebid Amoebozoa, and 46 archamoebids constructed from 1,052 unambiguously aligned characters, Archamoebae comprised a monophyletic group with a bootstrap support of 80 (Fig. 1). Within Archamoeba, the following three clades are well-supported as monophyletic groups: *Entamoeba*, bootstrap=100; a clade consisting of *Mastigamoeba*, *Endolimax*, and *Iodamoeba*, bootstrap=100; and *Pelomyxa*, bootstrap=100. *Rhizomastix libera* is sister to *Entamoeba* in a weakly supported clade (bootstrap=59). *Rhizomastix libera* and *Entamoeba* are sister to mastigamoebids.

Pelomyxids form a group sister to the rest of Archamoeba. The relative positions of *Pelomyxa* and *Mastigamoeba* switched when a 73 taxa subset was used instead of the 184 taxa dataset (Fig. 2). The basal branches in the 184 taxa tree were used for displaying an Archamoeba-only tree (Fig. 3).

2.4.3 Deepest Nodes in *Entamoeba*

Prior to adding cockroach-derived taxa, when vertebrate-inhabiting *Entamoeba* (VIE) were incorporated in trees with other Archamoebae, *E. polecki* was sister to all other *Entamoeba*. Generally, this was still the case after cockroach-derived taxa were added. This *E. polecki* position was recovered in the Amoebozoa + outgroups tree (Fig. 2), but not in an Archamoeba-only tree with 45 taxa (Fig. 3).

2.4.4 Occurrence

Entamoeba SSU sequences were detected by PCR and electrophoresis in all 34 *P. americana* specimens, the most heavily sampled species (Table 1). Sampling ranged from one to five specimens for each of the other seven species. *Entamoeba* were detected in at least one specimen of every host species. *Entamoeba* did not occur in two individuals of *C. punctulatus* and two of *B. germanica* (Table 2).

2.4.5 Relation of New Sequences to Sequences of Other *Entamoeba*

One hundred and five of the 110 sequences analyzed from *P. americana* belonged to a novel clade sister to the one predominated by vertebrate-inhabiting *Entamoeba* (Fig. 4). The novel clade was highly supported with a bootstrap of 100. The five *P. americana* sequences that did not belong to this clade grouped closely with *E. moshkovskii* (Fig. 11), an *Entamoeba* known previously from humans and anaerobic sediments (Tshalaia 1941; Scaglia 1983; Clark and Diamond 1991a; Ali et al. 2003). These sequences originated from two of the 11 *P. americana* individuals.

2.4.6 Clades and Patterns in New *Entamoeba* from All Sampled Cockroach Species

When *Entamoeba* sequences from all eight cockroach species were included in analyses, 11 distinct clades were observed, and three sequences (*Cryptocercus punctulatus* 1, *G. portentosa* 2-5, and *G. portentosa* 2-8) did not belong to any of these clades (Figs. 8 and 9). All 11 clades had at least two taxa and bootstrap support of at least 85. Some or all host specimens of *G. portentosa*, *B. germanica*, *B. orientalis*, and *P. pennsylvanica* contained multiple variants of *Entamoeba* (Table 2). No host specimens of *B. giganteus*, *P. fuliginosa*, and *C. punctulatus* had multiple variants. Sequences were retrieved from one to five individual hosts for each non-*P. americana* species. Host sampling was too low to make generalizations regarding the prevalence of monoinfection vs. mixed infection for these species, as well as generalizations regarding the diversity of *Entamoeba* in each host species. Of the 28 host specimens assessed, each host specimen has variants in one to six of the 11 clades (Fig. 9, Table 4). Each clade has

variants from one to fifteen host specimens and one to five host species (Figs. 8 and 9, Table 4). Each non-*P. americana* host species has variants in one to six clades. Two clades are exclusively non-*P. americana*, eight contain variants from *P. americana* and non-*P. americana*, and one is exclusively *P. americana*.

2.4.7 Clades in New *Entamoeba* and Patterns: *P. americana* only

When *Entamoeba* sequences from only *P. americana* were included, nine distinct clades were observed, and one sequence did not belong to any of these clades (Fig. 5). Sequences were highlighted according to host population (Fig. 5). Eight clades contained sequences from both the Carolina (laboratory) colony and wild population. The remaining clade contained sequences from only the wild population, though only a single host individual is represented in this clade.

All clades had more than two taxa and bootstrap support of at least 90. Most host specimens had variants in one to six of the nine clades (Fig. 6). Each clade had variants from one to six of the 11 *P. americana* host specimens.

Sexual identity was recorded for four host specimens (two males and two females) from which sequences were recovered. Sex was not associated with the number of clades represented (Fig. 7). It is clear that males and females can carry *Entamoeba* variants that belong to the same clade. Though sex-based differences in genetic diversity or phylogenetic patterns are not apparent, more sampling would be needed to assert that such differences do not exist.

2.4.8 Monophyly and Relationships of *Entamoeba* and other Archamoebae

We recovered Archamoeba as a highly supported monophyletic group in Amoebozoa, as did Ptackova et al. (2013), Stensvold et al. (2012), Fahrni et al. (2003), and Milyutina et al. (2001) in their molecular-based analyses. In my study, *Entamoeba* were recovered with maximum support both before and after addition of novel sequences.

The traditionally recognized families Mastigamoebidae, Pelomyxidae, and Entamoebidae were recovered as highly supported monophyletic clades (Figs. 2 and 3). Pelomyxids were a long branch sister to all other archamoebids. The group containing non-pelomyxid Archamoeba is weakly supported (bootstrap=34), and the grouping of *Rhizomastix* with *Entamoeba* is also weakly supported (bootstrap=68). Though the branching pattern here differs from that in Ptackova et al. (2013), the bootstrap values for those branches here are low and do not provide much additional support for the relative position of pelomyxids or *Rhizomastix* within Archamoeba.

2.4.9 Nodes in *Entamoeba*

Silberman et al. (1999) and Ptackova et al. (2013) recovered *Entamoeba coli* as sister to all other *Entamoeba* species. Prior to the addition of NVIE, a tree was constructed with 24 vertebrate-inhabiting *Entamoeba*, 17 non-*Entamoeba* Archamoeba, 110 non-archamoebid Amoebozoa, and 30 non-Amoebozoa. The *E. polecki* group was recovered as sister to the rest of *Entamoeba*. This position for *E. polecki* may have not been recovered in Silberman et al. (1999) because VIE sequences, primarily those discovered by Stensvold et al. (2011), were not available at the time. Other Archamoeba sequences such as *Rhizomastix libera*, *Pelomyxa* sp., and several

mastigamoebids were also not available then. The relationships within *Entamoeba* were not a major focus in Ptackova et al. (2013) and a high number of *Entamoeba* taxa were not included, possibly contributing to the position of *Entamoeba coli* as sister to the other *Entamoeba*. After addition of NVIE to a tree of VIE, non-*Entamoeba* Archamoeba, and non-Archamoeba taxa, *E. polecki* was still recovered as sister to all other *Entamoeba* (Fig. 2). No single group stood out as sister to the rest when trees were constructed with only Archamoeba (Fig. 3). This highlights the importance of conducting analyses with high taxon sampling when determining basal groups, and the caution with which taxa must be removed if a tree is being trimmed.

2.5 Discussion

2.5.1 Part 1

2.5.1.1 Occurrence

The infection rate was 100% in the two *P. americana* populations studied here, one which was wild and the other lab-reared (Table 1). At least one representative of the other seven host species tested were also infected, though too few specimens of these species were sampled to accurately estimate the infection rate within each species. Two of the cockroach species studied- *P. americana* and *B. orientalis*- have been previously reported to harbor *Entamoeba* (Hoyte 1961; Kidder 1937; Lucas 1927; Meglitsch 1940). These reports included descriptions of trophozoites, indicating that the *Entamoeba* were true inhabitants, rather than passive cysts. *Entamoeba* have also been reported from *B. germanica*, but these were reported as vectors of human-inhabiting *E. histolytica*, *E. dispar*, or *Entamoeba coli*, rather than hosts to true inhabitants (Fotedar et al. 1991; Kinfu and Erko 2008; Pai et al. 2003). It would be premature to

suggest that these scientists observed *Entamoeba* from the novel clade. Considering that only cysts were observed, it cannot be asserted that the observed *Entamoeba* exhibit active behavior in the cockroach. At the same time, identification using cyst morphology alone can easily result in failure to detect the full breadth of diversity- variants with similar cysts but different trophozoites or genotypes would be missed. This is a similar situation to uninucleate-encysters in humans, for which molecular data have revealed genetic diversity that was undetected in morphological identifications (Verweij et al. 2001).

Five of the cockroach species I screened are newly discovered hosts. These were *G. portentosa*, *B. giganteus*, *P. pennsylvanica*, *C. punctulatus*, and *P. fuliginosa*. The occurrence of *Entamoeba* in all eight species screened suggests they are likely to occur in many cockroaches that have not yet been screened. The sampled species are from four of the six traditional cockroach families (Blattidae, Blattellidae, Blaberidae, and Cryptocercidae), demonstrating that *Entamoeba* are found among multiple phylogenetic cockroach groups.

2.5.1.2 Relation of New Sequences to Sequences of Other *Entamoeba*

The major finding of this study is that the vast majority of cockroach-inhabitant sequences constitute a novel, highly supported clade that is sister to one predominated by VIE. Sequences for NVIE from the following hosts have also been retrieved in our lab (unpublished data): honeybee (*Apis mellifera*), green june beetle (larva, *Cotinis nitida*), Japanese beetle (larva, *Popillia japonica*), tipulid (larva, *Tipula* sp.), and giant water bug (*Belostoma* sp.). All belong to the novel clade except for those sequences from the giant water bug. For perspective of NVIE

diversity and to delineate groups, the novel clade was divided into sub-clades based on phylogenetic distance, high bootstrap support, and a minimum of two taxa per clade.

2.5.1.3 Clades and Patterns in New *Entamoeba* from All Sampled Cockroach Species

When only *Entamoeba* from *P. americana* were analyzed, they formed nine clades within the novel clade. When *Entamoeba* from the other seven cockroach species were added, many of them belonged to the nine clades and some of them formed an additional two clades. Looking at patterns of *Entamoeba* from *P. americana* first- before considering those from other cockroach hosts- was useful because it was the most extensively sampled host species and eliminated the variable of species. This allowed for comparison of variant diversity across host specimens and host populations (Figs. 5 and 6).

2.5.1.4 Monoinfection and Multiple Infection

Multiple *Entamoeba* variants were found in every *P. americana* specimen. For some specimens, these were distributed widely throughout the novel clade, in up to six clades, demonstrating that genetically diverse *Entamoeba* variants often inhabit a single individual of *P. americana* (Fig. 6, Table 4).

Rates of monoinfection and mixed infection varied across non-*P.americana* species. Sampling for these species was low, so it is inconclusive whether species that were only found to be monoinfected have the potential for mixed infection, or conversely, whether species that were only found to be mixed infected have monoinfected representatives in some situations.

2.5.1.5 Diversity

Based on branch lengths, number of distinct taxa, and number of sub-clades, *Entamoeba* from *P. americana* are about as genetically diverse as all previously known *Entamoeba* combined. This is the highest genetic diversity reported for *Entamoeba* from a single host species.

2.5.2 Discussion Part 2: Additional Discussion

2.5.2.1 Diversity

Many of the taxa from non-*P. americana* cockroaches belonged to clades containing *Entamoeba* from *P. americana*, while others constituted two new sub-clades. This relatively low number of additional sub-clades must be interpreted cautiously. As stated earlier, due to low sampling it would be premature to make statements regarding relative *Entamoeba* diversity per host species. If the same relative diversities hold at greater sample sizes, it would indicate that *P. americana* has a high genetic diversity of *Entamoeba* compared to other cockroach species. This does not seem unlikely, considering that *P. americana* is one of the most widespread cockroach species (Rueger and Olson 1969), inhabits a wide range of habitats, easily cohabits with other organisms (especially humans), and is highly aggregative (Roth 1973). These features could facilitate transmission of *Entamoeba* among conspecifics as well as between host species.

2.5.2.2 Occurrence

Originating in Africa, *P. americana* has spread to almost every human-inhabiting region of the world (Bell and Adiyodi 1981). The close human association allows them to live in

seemingly unsuitable places such as Alaska, where they would otherwise not survive away from the warmth and nourishment of human shelter (Rueger and Olson 1969). If the infection rate is high among other populations, as it is for those in our study, then cockroach-inhabiting *Entamoeba* may be the most widespread and common of all *Entamoeba*.

Insect guts have the potential to contain genetic material from food organisms, and cysts of organisms that are not active in cockroach guts might be found there. Detection of vectored microbes in cockroaches is one example of this (Fotedar et al. 1991; Kinfu and Erko 2008). Anytime sequences are retrieved without verification of the target organism by microscopy, the possibility exists that sequences are amplified from microbes that are not active inhabitants of the host. The *Entamoeba* in the novel clade recovered here are predicted to originate from true inhabitants because these sequences have not been found outside of insects, and because they were found among multiple cockroaches in multiple populations.

Determining whether reports of *E. moshkovskii* indicate passive or active inhabitants is difficult to determine. *Entamoeba moshkovskii* were detected in two *P. americana* in the present study, and were cultured in our lab from *P. americana* and *B. orientalis*. No other taxa we detected grouped among human-inhabiting species. Altogether, this suggests two possibilities: *E. moshkovskii* are human-inhabitants that are carried by cockroaches more frequently than other human-inhabitants, or *E. moshkovskii* are true cockroach-inhabitants. As far as I know, *E. moshkovskii* have never been unambiguously identified in cockroaches, even as a vectored microbe, prior to work in our lab. The lack of observed *E. moshkovskii* trophozoites in vector studies is not consistent with the second hypothesis. The status of *E. moshkovskii* as a true-inhabitant remains to be clarified, though I feel that such a status is likely, considering the

evidence from our lab and the wide range of temperature and osmotolerance that *E. moshkovskii* has been demonstrated to tolerate (Clark and Diamond 1997; Dreyer 1961; Richards et al. 1966).

2.5.2.3 Species

All 11 *P. americana* specimens from which sequences were collected had mixed infection, in that each housed multiple genetic variants of *Entamoeba*. Rates of mono and mixed infection varied across the other seven cockroach species. While mixed infection has been commonly reported in some vertebrates (Mukhopadhyay et al. 2002; Levecke et al. 2010), it has not been reported in non-vertebrates, largely because molecular work has not been conducted for them. There is wide sequence diversity in *Entamoeba* in a single cockroach specimen or species, but only one species of *Entamoeba* has been reported (in active, trophozoite form) in cockroaches (Lucas 1927). That all morphologically recognized *Entamoeba* in cockroaches have previously been classified as a single species is not surprising, as there are not a large number of easily visible morphological features for differentiation, and it can be difficult to determine whether observed *Entamoeba* are morphological variants within a single genetic lineage or among multiple genetic lineages. The frequent occurrence of mixed infection in cockroaches could mean that one lineage has not outcompeted another within the gut environment. This hints at the possibility of different genetic lineages fitting different ecological niches, and that the lineages are different in a biologically significant way (rather than simply genetically different). Non-morphological differences observed between VIE lineages include growth rate (Pysova et al. 2009), feeding behavior (Trissl et al. 1978), pathogenicity (Diamond and Clark 1993; Jaiswal et al. 2014), and social behavior (Espinosa and Paz-Y-Mino-C 2012).

Species of VIE have been distinguished based on isoenzyme analysis (Sargeant et al. 1982), RFLPs (Clark and Diamond 1991a,b), nuclei/cyst, pathogenicity, SSU rDNA, and host (Stensvold et al. 2010). Typically, a combination of these must be used for a convincing delineation of species (Clark and Diamond 1997; Verweij et al. 2001; Stensvold et al. 2011). In the current study, we only have SSU rDNA and host species, and have not observed sufficiently strong patterns of host specificity to suggest species. The data do not suggest host specificity for most variants. For the few variants that host specificity might be implicated, such as those in *G. portentosa*, host sampling was too low to confirm it. Some vertebrate species are host to multiple variants described as a single species, such as *E. bovis* in cows (Stensvold 2010; Stensvold et al. 2011) or *Entamoeba coli* in humans (Clark and Diamond 1997). These may be cases of genetically diverse species. These species definitions may be temporary, as variants may be described as multiple species after additional distinguishing characteristics are identified.

Producing mono-eukaryotic cultures of the variants would help in characterizing them and in delineating species. The cultures can be used to study morphology, behavior, and preferred environmental conditions. These characters could be linked to each genetic variant. Fluorescence in situ hybridization (FISH) could also be used for visualizing variants. In this method, fluorescent probes would be designed based on the SSU rDNA sequence of each variant and exposed to gut tissue. A fluorescence microscope would then be used to view the *Entamoeba*.

Currently, we do not have enough information to delineate species in the novel clade. Most of the clades contain *Entamoeba* from multiple host species, so defining each clade as a species based on host is not possible. The comparably high genetic diversity of the novel clade

relative to VIE species in the other clade suggests it is unlikely that the entire novel clade is a single species. If every variant in the clade is a new species, then there are 148 new species. While it would be extreme to claim that there are 148 new species, it is likely that each sub-clade consists of at least one new species, so there are probably at least 11 new species. The species taxonomy of *Entamoeba* will continue to change as more information, such as additional gene sampling and behavior, is gained and integrated into species characterization. The purpose of estimating possible species number at this point is to allow for some perspective of diversity of *Entamoeba* relative to other organisms. The morphology of *E. thomsoni* in cockroaches was described by Catherine Lucas in 1927. Most cysts she observed were 11-16 micrometers across, but some were eight micrometers. These cysts of different sizes could belong to various distinct lineages, and what would possibly be considered multiple species. The common occurrence of mixed infection in my study supports the likelihood that previously reported *E. thomsoni* consisted of multiple genetic variants. Although *E. thomsoni* may consist of multiple genetic variants, we do not have the necessary evidence to claim that *E. thomsoni* should be split into multiple species, because to do so would be based solely on molecular data, as these sequences have not yet been linked to particular morphologies.

2.5.2.4 *Entamoeba moshkovskii*

The discovery of *E. moshkovskii* in cockroaches has implications for species characterization and the possible role of cockroaches as vectors of this putative pathogen. *Entamoeba moshkovskii* has a broader host range than previously known. *Entamoeba moshkovskii* sequences from cockroaches were not exact duplicates as those retrieved from

Genbank, so it is possible that they are unique lineages with slight differences in SSU rDNA, not unlike *E. histolytica* and *E. dispar*. Or perhaps, *E. moshkovskii* are not biologically unique, but instead have the ability to switch environments. If *E. moshkovskii* in cockroaches can infect humans, and *E. moshkovskii* is pathogenic in humans, then cockroaches may be potential carriers of pathogenic *Entamoeba*, in a different way than they are for *E. histolytica*, which is not a true cockroach inhabitant. *Entamoeba moshkovskii* was found in two of 11 *P. americana*, and represents only five of the 85 unique, non-chimeric sequences collected from *P. americana*. In addition to being only occasionally detected in *P. americana*, it was also not detected in any of the other seven cockroach species screened. In our lab, *E. moshkovskii* has been cultured from *P. americana* and *B. orientalis* (Dr. Jeffrey Silberman, unpublished data). It is interesting that *E. moshkovskii* has grown in culture but that members of the new clade have not. Possible explanations for this observation include: 1) *E. moshkovskii* may have an advantage over other *Entamoeba* variants in the culture conditions, 2) *E. moshkovskii* is at relatively high numbers already compared to *Entamoeba* of the new clade, and 3) *E. moshkovskii* occurs in more cockroaches than is detected by PCR due to a molecular-level bias that favors amplification of particular variants. These possibilities are not mutually exclusive. Option 1 is difficult to address because we know so little about the habits of *Entamoeba* in the new clade. Option 2 is difficult to address because we do not have information about the number of each variant in *Entamoeba*. And option 3 is difficult to address without conducting experiments to determine these biases and/or collecting data using non-PCR methods for comparison.

2.5.2.5 Comparison of Populations

The two *P. americana* sources, Carolina Biological Supply Company and the University of Arkansas campus, differ in factors that could potentially effect transmission and maintenance of *Entamoeba*: Wild cockroaches are exposed to a greater variety of organisms than those raised in relatively non-biodiverse lab conditions. This could allow for transmission of *Entamoeba* from other host species. Wild cockroaches are also likely to have a different diet than what is provided in the lab, and diet has been shown to effect the microbial composition of the cockroach gut (Kane and Breznak 1991; Zurek and Keddie 1998; Bertino-Grimaldi et al. 2013). Transmission among conspecifics might also be effected by habitat type. The cockroaches from Carolina Supply were raised in limited enclosures at the company and in our lab, while those from the wild had virtually unlimited space. Captivity could have caused greater direct and fecal contact among conspecifics than would occur in the wild. On the other hand, *P. americana* still tend to aggregate in the wild, and may even aggregate more in response to extreme environmental conditions (Dambach and Goehlen 1999). It is likely that beyond a threshold level of exposure to conspecifics, additional exposure would not affect the genetic population structure of *Entamoeba* in cockroaches within a given population. My results so far support this hypothesis, as infection rates were equal between the populations, and both populations showed a similar spread of *Entamoeba* throughout the novel clade.

In this study, captivity did not seem to affect infection rate, which was 100% for both host populations. Overall, there was little difference in the number of clades to which *Entamoeba* from each population belonged, and most clades (eight of nine) contained variants from both host populations (Fig. 5). If more hosts were sampled from both populations, it is

possible that even the clade with only a single population represented would contain representatives from both populations. The representation of both host populations in most clades suggests that transmission may occur easily, and *Entamoeba* from different clades and host populations are not geographically isolated. Generally, the host species *P. americana* does not show much geographical isolation. Since beginning to spread from Africa 400 years ago, *P. americana* now occur as globally as the species (humans) they cohabit with (Bell and Adiyodi 1981). Molecular studies of human-associated cockroaches show high genetic variability and the existence of sub-populations, but with moderate to high gene flow between them. Pechal et al. (2008) assessed gene flow among *P. americana* populations by comparison of ITS1 (internal transcribed spacer 1), a region of DNA found between rRNA coding-regions and having a higher rate of change. The majority of samples were collected from a single college campus in Texas, but a few were from cities 8 to 462 km away. The data revealed that populations were not isolated and that interbreeding was common. Cloarec et al. (1999) assessed genetic variability of *B. germanica* within and between populations by comparisons of eight gene loci. Cockroaches from two French cities 900 km apart were sampled. Populations within each city were highly genetically differentiated, but the total populations of the two cities were not. These studies on cockroach population structure are consistent with the notion that *Entamoeba* are transmitted between populations frequently enough that geographic isolation of these *Entamoeba* would not be detected by analysis of SSU rDNA, because the rate of change of SSU rDNA is likely lower than the frequency of host migration between populations. The lack of geographical isolation supports my finding that cockroaches from two populations frequently have *Entamoeba* that belong to the same clade.

Housing did not appear to effect sequence diversity in cockroaches. It may be that *P. americana* were already sharing *Entamoeba* so frequently in the wild that placing them in more crowded conditions did not further increase transmission. The encystment capability of *Entamoeba* may make this hypothesis more likely for *Entamoeba* than for non-encysting microbes.

2.5.2.6 Host-Entamoeba Relationship over Evolutionary Time

Most cockroach-inhabiting *Entamoeba* sequences belong in a clade distinct from the primarily vertebrate-inhabitant clade, suggesting that the common ancestor of all cockroach-inhabiting *Entamoeba* was an inhabitant of the LCA of cockroaches. The less parsimonious scenario would involve transfer to multiple cockroach lineages after host divergence from an LCA.

Comparison of host phylogeny and symbiont phylogeny can be used to show vertical transmission of a microbe through multiple lineages. A close match would suggest that the microbe was present in the LCA of the extant hosts, with little to no horizontal transfer between host lineages following the LCA. This type of tight cospeciation has been found for *Blattabacterium* and cockroaches across multiple host families, and between *Blattabacterium* and the cockroach *Cryptocercus* at the level of host species and host strain (Lo et al. 2003; Clark et al. 2001). *Blattabacterium* inhabits the cockroach's fat bodies and is transmitted transovarially (Donnellan and Kilby 1967; Wren et al. 1989). Horizontal transfer is expected to be less likely for an intracellular symbiont such as *Blattabacterium* than for a gut-inhabitant, which could be more exposed to the outside environment. In our study, most of the sub-clades of *Entamoeba*

from cockroaches were not exclusively associated with specific host species, suggesting that many of these *Entamoeba* variants were transferred between host lineages at points throughout their evolutionary history.

Phylogeny in relation to host has been studied for *Nyctotherus* and *Blattabacterium* in non-Cryptocercid cockroaches too (Van Hoek et al. 1998; Clark et al. 2001; Lo et al. 2003). These provide interesting studies for comparison. The lack of evidence for cospeciation for *Entamoeba* in cockroaches shows more similarity to host-microbe phylogeny in *Nyctotherus* than in *Blattabacterium*. The tight cospeciation in *Blattabacterium* is likely due to its vertical mode of transmission, while a gut microbe such as *Nyctotherus* is in an environment more confluent with the outside environment, potentially permitting less discriminate host transfer. Both *Nyctotherus* and *Entamoeba* encyst. However, the patterns of infection for *Entamoeba* differ from those of *Nyctotherus* in significant ways. Van Hoek et al. (1998) did not find mixed infection for *Nyctotherus* in individual cockroach specimens, or even within a strain (only one ribotype/strain). One ribotype was found in 2 *Blaberus* sp. and 1 *P. americana* strain (Van Hoek et al. 1998). For *Entamoeba* in cockroaches, there are clades that contain *Entamoeba* from both *Blaberus* and *P. americana*, as well as *Entamoeba* from other host species. This suggests that there may be greater host switching and/or less host specificity among cockroach-inhabiting *Entamoeba* compared to *Nyctotherus*. Many clades also contain both wild and Carolina strains. Altogether, this suggests that host transfer has occurred among multiple cockroach strains and species, but has not occurred between cockroach and vertebrates. The one exception to this pattern is *E. moshkovskii*, which can inhabit both humans and *P. americana*.

2.5.2.7 Host-Cockroach Relationship and Vector Potential

The nature of the relationship between *Entamoeba* and cockroaches is mostly unknown, though some clues can be gleaned from current knowledge. It is likely that *Entamoeba* in the novel clade require invertebrate hosts, as *Entamoeba* in the novel clade have only been found in invertebrates. It is likely that they are true inhabitants rather than passers-through because these sequences have never been found outside of cockroaches. If these sequences had been found in many kinds of hosts, it would be unclear which organism was the true host, and would also allow the possibility that they might be commonly vectored through non-host organisms. However, the finding of such a high infection rate in *P. americana* with novel *Entamoeba* sequences suggests the opposite.

Entamoeba moshkovskii was found in two cockroach specimens. This species is a putative pathogen in humans (Shimokawa et al. 2012). Whether it has a pathogenic lifestyle in cockroaches remains to be studied. Pathogenicity is currently unknown for NVIE, though it has not been specifically studied.

The detection of *Entamoeba* in all 34 *P. americana* specimens, and the survival of our lab colony through multiple generations indicates that at least some variants, if not all of them, are not lethally pathogenic. It is likely that at least some of these probably feed on bacteria or partially digested food rather than directly on cockroach tissue. A symbiotic relationship can also not be ruled out. In other studies, administration of metronidazole to cockroaches resulted in elimination of *Nyctotherus ovalis* and methanogenesis (Bracke et al. 1978; Gijzen et al. 1991). Metronidazole is commonly used as a medical treatment for *Entamoeba*, and would probably have eliminated *Entamoeba* from cockroaches as well in these experiments. It is possible that

some of the inhibition of methanogenesis resulted from elimination of methanogen-housing *Entamoeba*. These methanogens might provide nutritious compounds to cockroaches (Kane and Breznak 1991), so if these *Entamoeba* house methanogens, their presence may benefit the host and add selective pressure on the cockroach to maintain *Entamoeba*. It is also possible that by effecting bacterial populations and diversity in the gut, presumably by grazing, these *Entamoeba* indirectly influence cockroach health. Multiple studies support the premises of this assertion. He et al. (2012) found that hindgut microbial diversity in rabbits changed significantly upon *E. histolytica* infection. Effects of the gut microbiota on insect health have also been demonstrated. For example, administration of lactic acid bacteria to bees resulted in increased infection resistance and decreased mortality (Vasquez et al. 2012). The importance of *Entamoeba* to cockroach health could be measured by procuring *Entamoeba*-free cockroaches and then re-introducing *Entamoeba*. To get *Entamoeba*-free cockroaches, cleaned eggs could be raised in an *Entamoeba*-free environment, or the drug metronidazole could be administered to adults to eliminate anaerobes (including *Entamoeba*) from the gut. Reintroduction could be achieved by feeding the cockroaches *Entamoeba* in monoeukaryotic culture or cysts separated from the feces using floatation methods. The reintroduction step would be important for distinguishing the health effects of just *Entamoeba* from those of other gut microbes.

Entamoeba in cockroaches have been overlooked, even though they may constitute a large portion of biodiversity in the genus. The high genetic diversity of these *Entamoeba* suggests that there are probably multiple species of *Entamoeba* in cockroaches, even though

these species may currently appear "cryptic" due to a lack of information on other biologically relevant aspects such as morphology and behavior. The prevalence of infection in *P. americana*, as well as across eight species of cockroaches that span much of Blattodean phylogeny, highlights the widespread occurrence of *Entamoeba* that have been absent from current ecological and phylogenetic inventories.

Species (Scientific Name)	Species (Common Name)	Specimens Screened	Specimens with <i>Entamoeba</i>	Specimens with Sequences
<i>P. americana</i>	American cockroach	34	34	11
<i>B. germanica</i>	German cockroach	5	3	1
<i>B. giganteus</i>	Giant cockroach	4	4	1
<i>B. orientalis</i>	Oriental cockroach	3	3	3
<i>C. punctulatus</i>	Wood-eating cockroach	3	1	1
<i>G. portentosa</i>	Madagascar hissing cockroach	5	5	5
<i>P. pennsylvanica</i>	Pennsylvania wood cockroach	5	5	5
<i>P. fuliginosa</i>	Smokybrown cockroach	1	1	1
Total		60	56	28

Table 1. Summary of Host Sampling. "Specimens with *Entamoeba*" specifies the number of cockroaches with 1600 bp bands in electrophoresis following PCR with *Entamoeba*-specific primers. "Specimens with Sequences" specifies the number of cockroaches from which SSU rDNA sequences were recovered for phylogenetic analysis.

Species	Specimen Name	Source	Dissection Date	Mono infection (grey-white), mixed infection (grey-grey), or N/a or unknown (white)	<i>Entamoeba</i> SSU band detected (grey) or not (white)	Abbreviation if sequenced	Sequences Collected	Sequences in alignment	Non-chimeric unique hindgut	Non-chimeric unique non-hindgut	Colonies Collected	Colonies positive for <i>Entamoeba</i> based on 1F1700R	# of unique RFLPs
<i>P. americana</i>	NPA	Carolina	2010			NPA21, NPA 22	2	2	1	0	10+	Unknown	Unknown
<i>P. americana</i>	Pa1	Carolina	2010			Pa1	14	14	8	0	10+	Unknown	Unknown
<i>P. americana</i>	Pa2	Carolina	2010			Pa2	8	8	8	0	10+	Unknown	Unknown
<i>P. americana</i>	Peri 1	Carolina	08/30/10			Pa1-#or	18	18	12	6	94	62	8
<i>P. americana</i>	Peri 3	Outside by Bell Engineering	10/04/10			Pa3-#or	36	36	10	23	101 from rectum, colon, ventriculus, and proventriculus. 98 from caeca, crop, fat, and head.	91 from rectum, colon, ventriculus, and proventriculus. 86 from caeca, crop, fat, and head.	13 from rectum, colon, ventriculus, and proventriculus. 11 from caeca, crop, fat, and head. (13 and 11 not compared to each other, so may be overlap)
<i>P. americana</i>	Peri 4	Carolina	11/22/10				0	0	0	0	0	0	0
<i>P. americana</i>	Peri 5	University Recreation Center (HPER), Men's Locker room	12/24/10			Pa5	10	10	10	0	30+	32	10
<i>P. americana</i>	Peri 6	Carolina	01/19/11			Pa4	20	20	19	0	30+	37	20
<i>P. americana</i>	Peri 7	Outside by Bell Engineering	06/09/11				0	0	0	0	30+	0	0
<i>P. americana</i>	Peri 8	Outside by Bell Engineering	06/09/11			Pa7	8	8	8	0	0	14	5
<i>P. americana</i>	Peri 9	Outside by Bell Engineering	06/11/11			Pa8	8	8	8	0	0	13	7
<i>P. americana</i>	Peri 10	Carolina	08/03/11				0	0	0	0	0	0	0
<i>P. americana</i>	Peri 11	Carolina	08/03/11				0	0	0	0	0	0	0
<i>P. americana</i>	Peri 12	Outside by Bell Engineering	08/04/11				0	0	0	0	0	0	0
<i>P. americana</i>	Peri 13	Carolina	08/04/11				0	0	0	0	0	0	0
<i>P. americana</i>	Peri 14	Carolina	08/04/11				0	0	0	0	0	0	0
<i>P. americana</i>	Peri 15	Outside by Bell Engineering	08/05/11				0	0	0	0	0	0	0
<i>P. americana</i>	Peri 16	Outside by Bell Engineering	08/05/11				0	0	0	0	0	0	0
<i>P. americana</i>	Peri 17	Carolina	08/05/11				0	0	0	0	0	0	0
<i>P. americana</i>	Peri 18	Carolina	08/06/11				0	0	0	0	0	0	0
<i>P. americana</i>	Peri 19	Carolina	08/07/11				0	0	0	0	0	0	0
<i>P. americana</i>	Peri 20	Carolina	08/08/11				0	0	0	0	0	0	0
<i>P. americana</i>	Peri 21	Carolina	08/09/11				0	0	0	0	0	0	0
<i>P. americana</i>	Peri 22	Carolina	08/09/11				0	0	0	0	0	0	0
<i>P. americana</i>	Peri 23	Outside by Bell Engineering	08/10/11				0	0	0	0	0	0	0
<i>P. americana</i>	Peri 24	Outside by Bell Engineering	08/10/11			Pa24	12	12	11	0	40	39	5
<i>P. americana</i>	Peri 25	Outside by Bell Engineering	08/11/12				0	0	0	0	0	0	0

Table 2. Details of dissected cockroaches and *Entamoeba* sequences.

Species	Specimen Name	Source	Dissection Date	Mono infection (grey-white), mixed infection (grey-grey), or N/a or unknown (white)	<i>Entamoeba</i> SSU band detected (grey) or not (white)	Abbreviation if sequenced	Sequences Collected	Sequences in alignment	Non-chimeric unique hindgut	Non-chimeric unique non-hindgut	Colonies Collected	Colonies positive for <i>Entamoeba</i> based on 1F1700R	# of unique RFLPs
<i>P. americana</i>	Peri 26	Outside by Bell Engineering	08/13/11				0	0	0	0	0	0	0
<i>P. americana</i>	Peri 27	Outside by Bell Engineering	08/15/11				0	0	0	0	0	0	0
<i>P. americana</i>	Peri 28	Outside by Bell Engineering	08/15/11				0	0	0	0	0	0	0
<i>P. americana</i>	Peri 29	Outside by Bell Engineering	08/17/11				0	0	0	0	0	0	0
<i>P. americana</i>	Peri 30	Outside by Bell Engineering	08/18/11				0	0	0	0	0	0	0
<i>P. americana</i>	Peri 31	Outside by Bell Engineering	08/18/11				0	0	0	0	0	0	0
<i>P. americana</i>	Peri 32	Ferritor Lab Building, Men's urinal	10/07/11			Pa32	4	4	2	0	20	16	7
<i>B. germanica</i>	Blat 2	632 W. Putman., Fayetteville, AR	11/22/10			Bge2	13	13	7	2	69	61	10
<i>B. germanica</i>	Blat 3	Carolina	03/02/11				0	0	0	0	0	0	0
<i>B. germanica</i>	Blat 4	Courtney C.'s Apt., Fayetteville, AR	05/01/11				0	0	0	0	0	0	0
<i>B. germanica</i>	Blat 5	Courtney C.'s Apt., Fayetteville, AR	05/09/11				0	0	0	0	0	0	0
<i>B. germanica</i>	Blat 6	Courtney C.'s Apt., Fayetteville, AR	05/09/11				0	0	0	0	0	0	0
<i>B. giganteus</i>	Blab 1	Carolina	02/04/11			Bgi1	9	9	2	6	109	35	8
<i>B. giganteus</i>	Blab 2	Carolina	02/22/11				0	0	0	0	0	0	0
<i>B. giganteus</i>	Blab 3	Carolina	04/21/11				0	0	0	0	0	0	0
<i>B. giganteus</i>	Blab 4	Carolina	04/23/11				0	0	0	0	0	0	0
<i>G. portentosa</i>	Grom 1	Steinkraus	11/23/11			Gp1	1	1	1	0	Only one variant. no cloning	0	0
<i>G. portentosa</i>	Grom 2	Steinkraus	11/29/11			Gp2	5	5	3	0	23	20	8
<i>G. portentosa</i>	Grom 3	Steinkraus	11/29/11			Gp1	1	same as Gp1	0	0	Only one variant. no cloning	0	0
<i>G. portentosa</i>	Grom 4	Steinkraus	11/29/11			Gp1	1	same as Gp1	0	0	Only one variant. no cloning	0	0
<i>G. portentosa</i>	Grom 5	Steinkraus	11/29/11			Gp1	1	same as Gp1	0	0	Only one variant. no cloning	0	0
<i>B. orientalis</i>	Ori 1	Outside by Bell Engineering	06/15/11			Bo1	1	1	1	0	Only one variant. no cloning	0	0
<i>B. orientalis</i>	Ori 2	Outside by Bell Engineering	06/15/11			Bo2	2	2	1	0	7	7	3
<i>B. orientalis</i>	Ori 3	Outside by Bell Engineering	06/15/11			Bo3	7	7	5	0	23	23	7
<i>C. punctulatus</i>	Cryp 1	Gatlinburg, TN, Smokey Mtns	07/20/11			Cp1	1	1	1	0	Only one variant. no cloning	0	0
<i>C. punctulatus</i>	Cryp 2	Gatlinburg, TN, Smokey Mtns	07/20/11				0	0	0	0	0	0	0
<i>C. punctulatus</i>	Cryp 3	Gatlinburg, TN, Smokey Mtns	07/20/11				0	0	0	0	0	0	0
<i>P. fuliginosa</i>	Ful 1	2200 W. Holly, Fayetteville, AR, indoor, by door	05/08/12			Pf1	1	1	1	0	Only one variant. no cloning	0	0
<i>P. pennsylvanica</i>	Parc 1	House in Woods, windowsill	06/07/11			Pp1	1	1	1	0	30	30	3
<i>P. pennsylvanica</i>	Parc 12	UA Farm	05/04/12			Pp12	2	2	2	0	30	30	2
<i>P. pennsylvanica</i>	Parc 16	UA Farm	05/04/12			Pp16	2	2	2	0	30	30	3
<i>P. pennsylvanica</i>	Parc 17	UA Farm	05/06/12			Pp17	3	3	3	0	30	27	7
<i>P. pennsylvanica</i>	Parc 22	2200 W Holly Fayetteville, AR, indoor, by door	05/08/12			Pp22	5	5	5	0	24	23	4

Table 2. Continued.



Fig. 1. Phylogeny with selected *Entamoeba*, Amoebozoa, and non-Amoebozoan outgroups. Maximum-likelihood with GTR+GAMMA was used to assess relationships between 184 taxa using 1,052 aligned characters. Bootstrap supports of 80 and above within Archamoeba are indicated.

Non-Amoebzoa
<i>Candida maltosa</i>
<i>Phytophthora palmivora</i>
<i>Toxoplasma gondii</i>
<i>Eimeria tenella</i>
<i>Mallomonas rasilis</i>
<i>Oryza sativa</i>
<i>Chlamydomonas reinhardtii</i>
<i>Cyanophora paradoxa</i>
<i>Goniomonas truncata</i>
<i>Guillardia theta</i>
<i>Cafeteria roenbergensis</i>
<i>Porphyra yezoensis</i>
<i>Phaeocystis globosa</i>
<i>Emiliana huxleyi</i>
<i>Glaucocystis nostochinearum</i>
<i>Cyanidium caldarium</i>
<i>Galeidinium rugatum</i>
<i>Gymnodinium microreticulatum</i>
<i>Jakoba libera</i>
<i>Reclinomonas americana</i>
<i>Platyreta germanica</i>
<i>Cercomonas longicauda</i>
<i>Apusomonas proboscidea</i>
<i>Ancyromonas sigmoides strain</i>
<i>Mnemiopsis leidyi</i>
<i>Dermocystidium salmonis</i>
Non-Archamoeba Amoebzoa
<i>Athelia bombacina</i>
<i>Hartmannella cantabrigiensis</i>
<i>Hartmannella vermiformis</i>
<i>Echinamoeba exundans</i>
<i>Paraflabellula hoguae</i>
<i>Heleopera sphagni</i>
<i>Centropyxis laevigata</i>
<i>Leptomyxa reticulata</i>

Table 3. List of taxa used in tree provided in Figure 1.

Non-Archamoeba Amoebozoa (continued)
Saccamoeba limax
Glaeseria mira
Chaos nobile
Chaos carolinense
Balamuthia mandrillaris (isolate V039)
Balamuthia mandrillaris
Protosteliales sp. LHI05
Protacanthamoeba bohémica
Acanthamoeba astronyxis
Acanthamoeba pearcei
Acanthamoeba castellanii
Acanthamoeba tubiashi
Sappinia diploidea
Sappinia pedata
Nematostelium ovatum
Schizoplasmodium cavostelioides
Ceratiomyxella tahitiensis isolate
Thecamoeba similis
Thecamoeba quadrilineata
Platyamoeba stenopodia
Endostelium zonatum
Dermamoeba algensis
Mayorella sp. JJP-2003
Multicilia marina
Phalansterium solitarium
Gephyramoeba sp. ATCC50654
Soil amoeba AND16
Lobosea sp. Borok
uncultured eukaryote clone RT5iin44
Filamoeba sinensis
Filamoeba nolandi
Planoprotostelium aurantium
Protostelium okumukumu
Protostelium nocturnum
Tychosporium acutostipes strain KE11AL
Tychosporium acutostipes strain NZ05-15a
Schizoplasmodiopsis pseudoendospora
Soliformovum irregulare
Soliformovum expulsum
Schizoplasmodiopsis amoeboides strain BG7A-12B

Table 3. Continued.

Non-Archamoeba Amoebozoa (continued)
<i>Schizoplasmodiopsis amoeboidea</i> culture-collection ATCC:46943
<i>Cavostelium apophysatum</i>
<i>Dictyostelium discoideum</i>
<i>Dictyostelium purpureum</i>
<i>Dictyostelium</i> sp. <i>menorah</i>
<i>Dictyostelium minutum</i>
<i>Actyostelium ellipticum</i>
<i>Polysphondylium pallidum</i>
<i>Dictyostelium deminutivum</i>
<i>Dictyostelium bifurcatum</i>
<i>Arcyria denudata</i>
<i>Trichia persimilis</i>
<i>Trichia decipiens</i>
<i>Lindbladia tubulina</i>
<i>Symphytocarpus impexus</i>
<i>Stemonitis flavogenita</i>
<i>Physarum polycephalum</i>
<i>Protophysarum phloiogenum</i>
<i>Didymium nigripes</i>
<i>Clastostelium recurvatum</i>
<i>Protosporangium articulatum</i>
<i>Vannella anglica</i>
<i>Vannella</i> sp. strain ISO13/I
<i>Platyamoeba contorta</i> isolate W51C#4
<i>Platyamoeba placida</i>
<i>Protosteliopsis fimicola</i> culture-collection CCAP:1569/I clone 3
<i>Vannella</i> sp. strain 4362V/II
<i>Platyamoeba plurinucleolus</i> strain ATCC 50745
<i>Vannella</i> sp. strain RSL/I
<i>Lingulamoeba leei</i>
<i>Vannella</i> sp. strain CAZ6/I
<i>Vannella</i> sp. strain W181G/I
<i>Clydonella</i> sp. ATCC 50884
<i>Clydonella</i> sp. ATCC 50816
<i>Korotnevella hemistylolepis</i>
<i>Vexillifera armata</i>
<i>Neoparamoeba pemaquidensis</i>
<i>Neoparamoeba aestuarina</i> isolate SL200
<i>Neoparamoeba branchiphila</i>

Table 3. Continued.

Non-Archamoeba Amoebozoa (continued)
<i>Korotnevella stella</i>
<i>Paramoeba eilhardi</i>
<i>Labyrinthulid quahog parasite QPX</i>
<i>Diaphanoeca grandis</i>
<i>Euglypha rotunda</i>
<i>Protosteliopsis fimicola</i> strain Ken-A
<i>Protosteliopsis fimicola</i> strain OM05-6218-1
<i>Protosteliopsis fimicola</i> culture-collection CCAP:1569/I clone 4
<i>Protosteliales</i> sp. LHI05
Archamoeba
<i>Entamoeba histolytica</i>
<i>Entamoeba coli</i> strain HU-1:CDC
<i>Mastigamoeba guttula</i>
<i>Iodamoeba</i> sp. RL2 isolate EM080 clone I
<i>Iodamoeba</i> sp. RL1 isolate 1074
<i>Mastigamomeba</i> sp. ATCC50617
<i>Mastigamoeba errans</i>
<i>Mastigamoeba abducta</i> isolate CHOM1 clone 1_1
<i>Mastigella commutans</i>
<i>Phreatamoeba balamuthi</i>
<i>Mastigamoeba lenta</i> isolate VIT1AN
<i>Mastigamoeba simplex</i>
<i>Endolimax nana</i>
<i>Rhizomastix libera</i> isolate IND8MA clone 1_8
<i>Entamoeba struthionis</i>
<i>Entamoeba equi</i>
<i>Entamoeba gingivalis</i>
<i>Entamoeba suis</i>
<i>Entamoeba muris</i>
<i>Entamoeba ranarum</i>
<i>Entamoeba invadens</i>
<i>Entamoeba terrapinae</i>
<i>Entamoeba</i> sp. NIH:1091:1
<i>Entamoeba coli</i> strain HU-1:CDC
<i>Entamoeba histolytica</i>
<i>Entamoeba dispar</i>
<i>Entamoeba</i> sp. CS-2010

Table 3. Continued.

Archamoeba (continued)
<i>Entamoeba</i> sp. RL3 partial 18S rRNA gene, isolate 09/1247
<i>Entamoeba bovis</i>
<i>Entamoeba coli</i>
<i>Entamoeba polecki</i>
<i>Entamoeba hartmanni</i>
<i>Entamoeba insolita</i>
<i>Entamoeba chattoni</i>
<i>Entamoeba</i> sp. RL2 partial
<i>Entamoeba moshkovskii</i>
Pa2-8
Pa7-40
Pa1-9co
Pa1-5
Pa5-23
Pa3-3re
Pa4-13

Table 3. Continued.

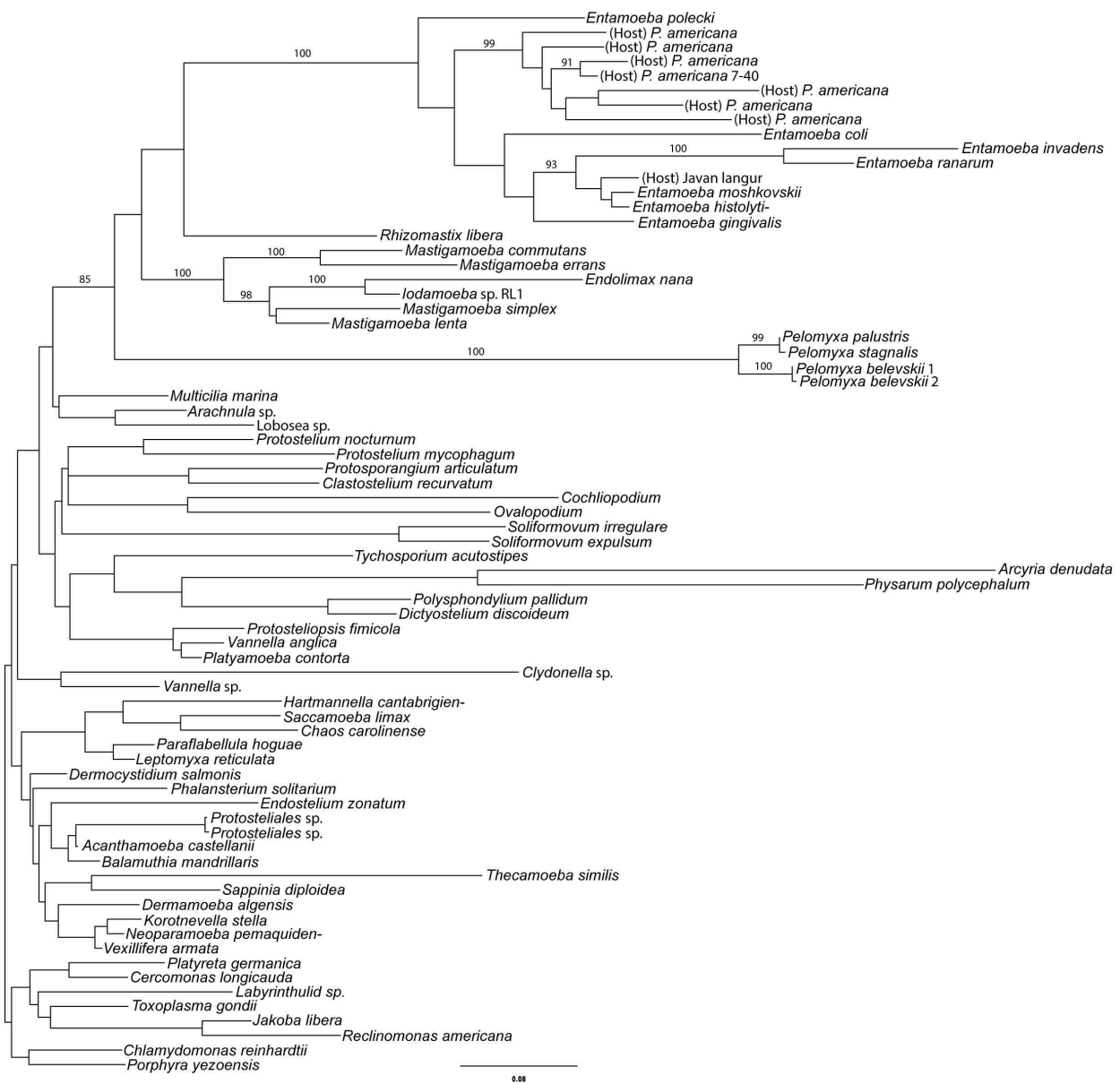


Fig. 2. An unrooted phylogeny of Archamoeba, non-Archamoeba Amoebozoa, and non-Amoebozoa. Maximum-likelihood with GTR+GAMMA was used to assess relationships between 73 taxa using 1,052 aligned characters. Bootstrap values are indicated for Archamoeba and for clades within it when above 90.

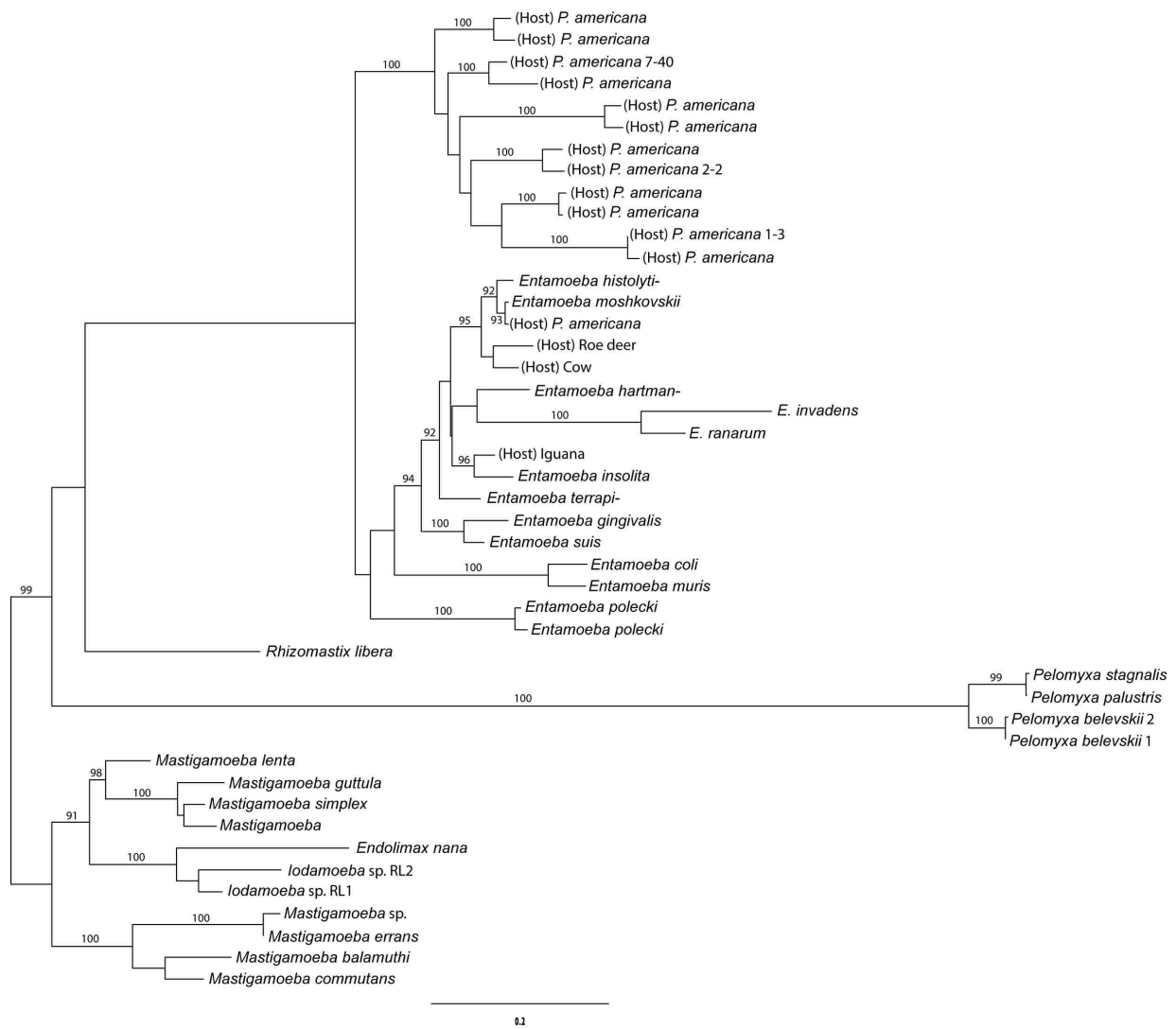


Fig. 3. An unrooted phylogeny of Archamoeba. Maximum-likelihood with GTR+GAMMA was used to assess relationships of 45 taxa using 1,119 aligned characters. Bootstrap values are indicated when above 90.

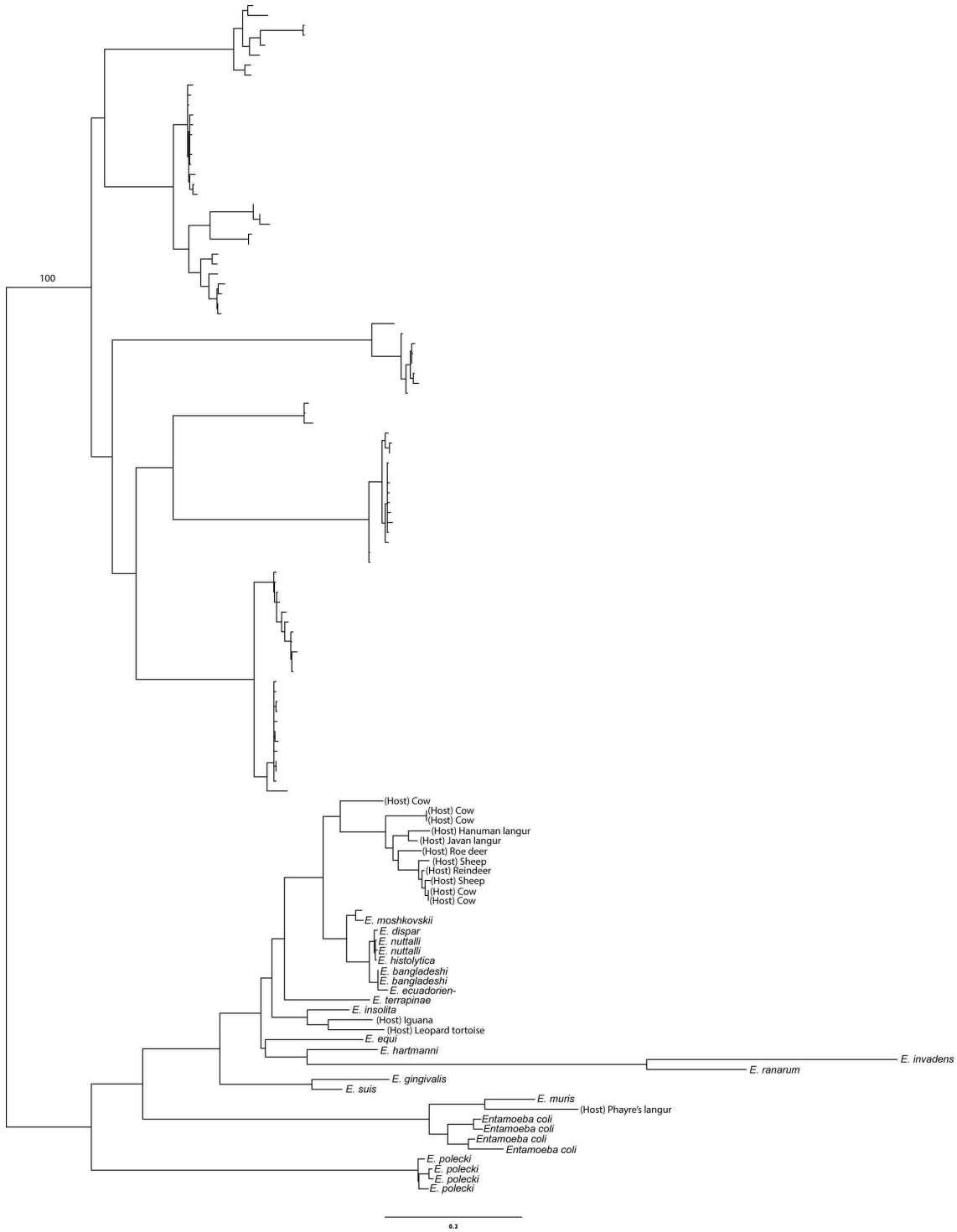


Fig. 4. An unrooted phylogeny of *Entamoeba* from *P. americana* hindgut and vertebrates. Unlabelled tips indicate taxa from *P. americana* hindgut. Maximum-likelihood with GTR+GAMMA was used to assess relationships between 120 taxa using 1,272 aligned characters.

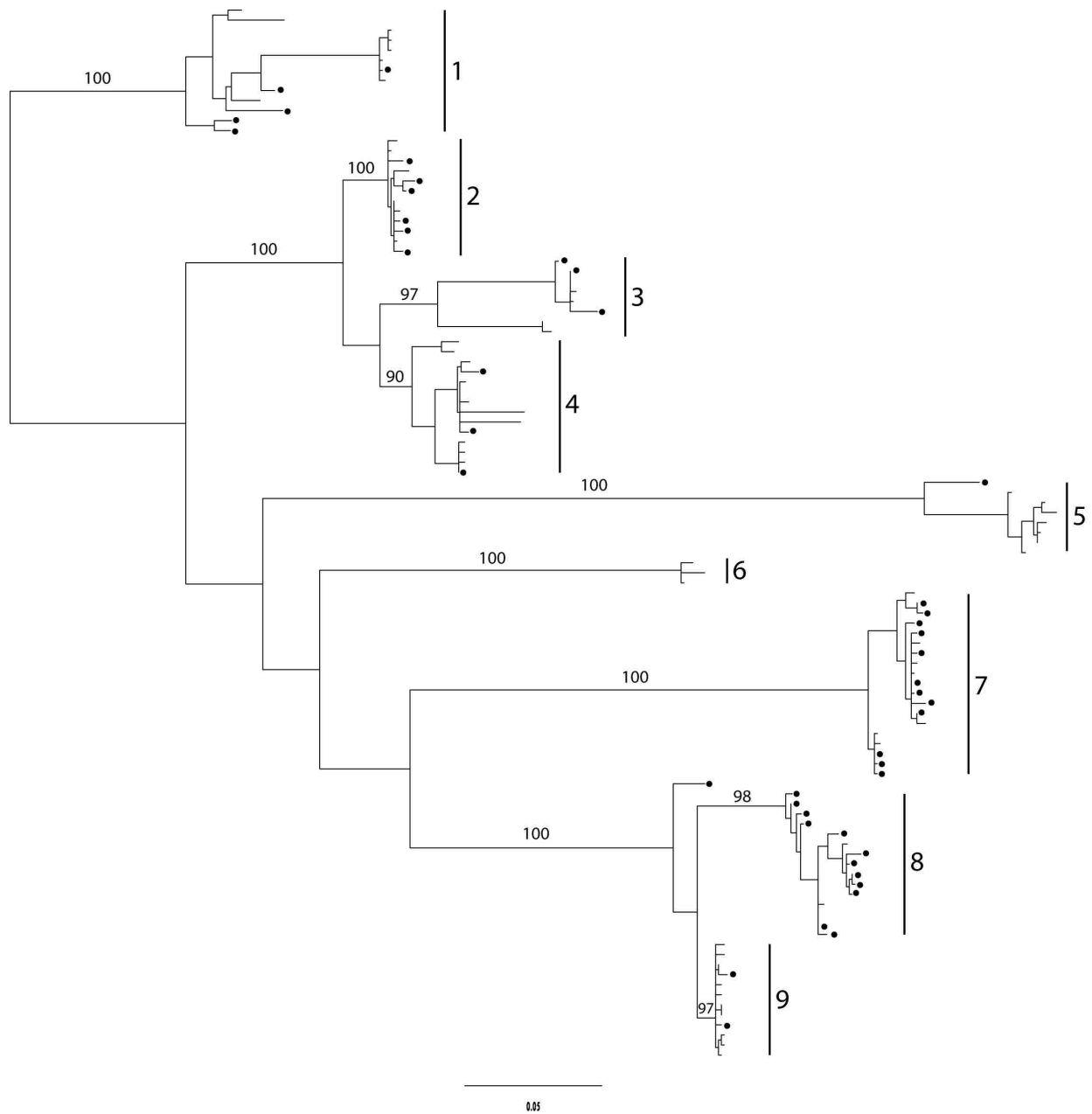


Fig. 5. An unrooted phylogeny of *Entamoeba* SSU rDNA from *P. americana*. Major sub-clades are numbered. Branch tips with circles indicate taxa from the Carolina (lab) host population. Branch tips without circles indicate taxa from the wild host population. Bootstrap values are indicated for the major sub-clades. Maximum-likelihood with GTR+GAMMA was used to assess relationships between 105 taxa using 1,272 aligned characters.

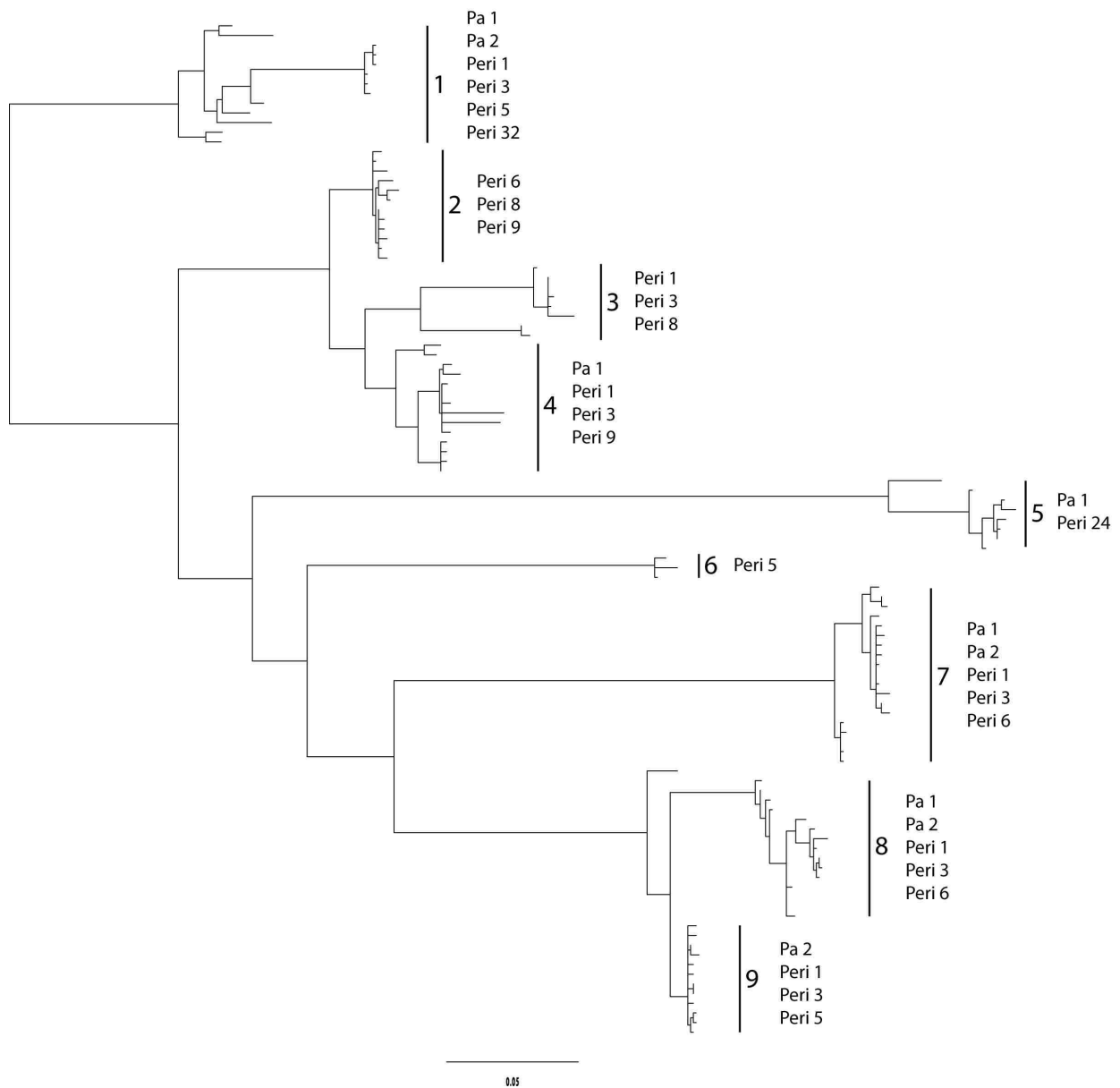


Fig. 6. An unrooted phylogeny of *Entamoeba* SSU rDNA from *P. americana*. Major sub-clades are numbered. Host specimens with *Entamoeba* in the sub-clade are indicated. Maximum-likelihood with GTR+GAMMA was used to assess relationships between 105 taxa using 1,272 aligned characters.

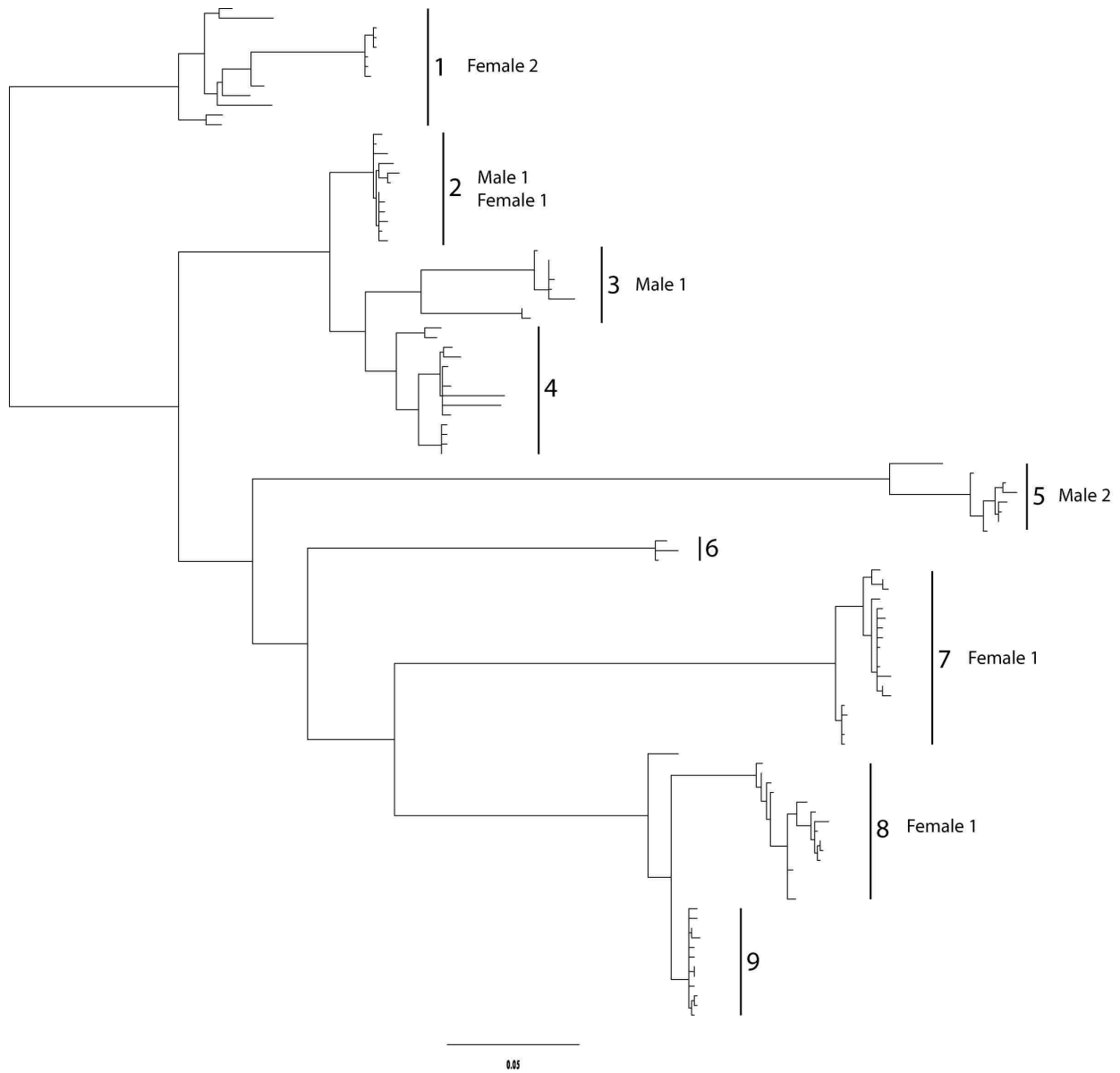


Fig. 7. An unrooted phylogeny of *Entamoeba* SSU rDNA from *P. americana*. Major sub-clades are numbered. Two host males and 2 host females are indicated when they have *Entamoeba* in sub-clades. Male 1= Peri 8, Male 2= Peri 24, Female 1= Peri 6, Female 2= Peri 32. Maximum-likelihood with GTR+GAMMA was used to assess relationships between 105 taxa using 1,272 aligned characters.

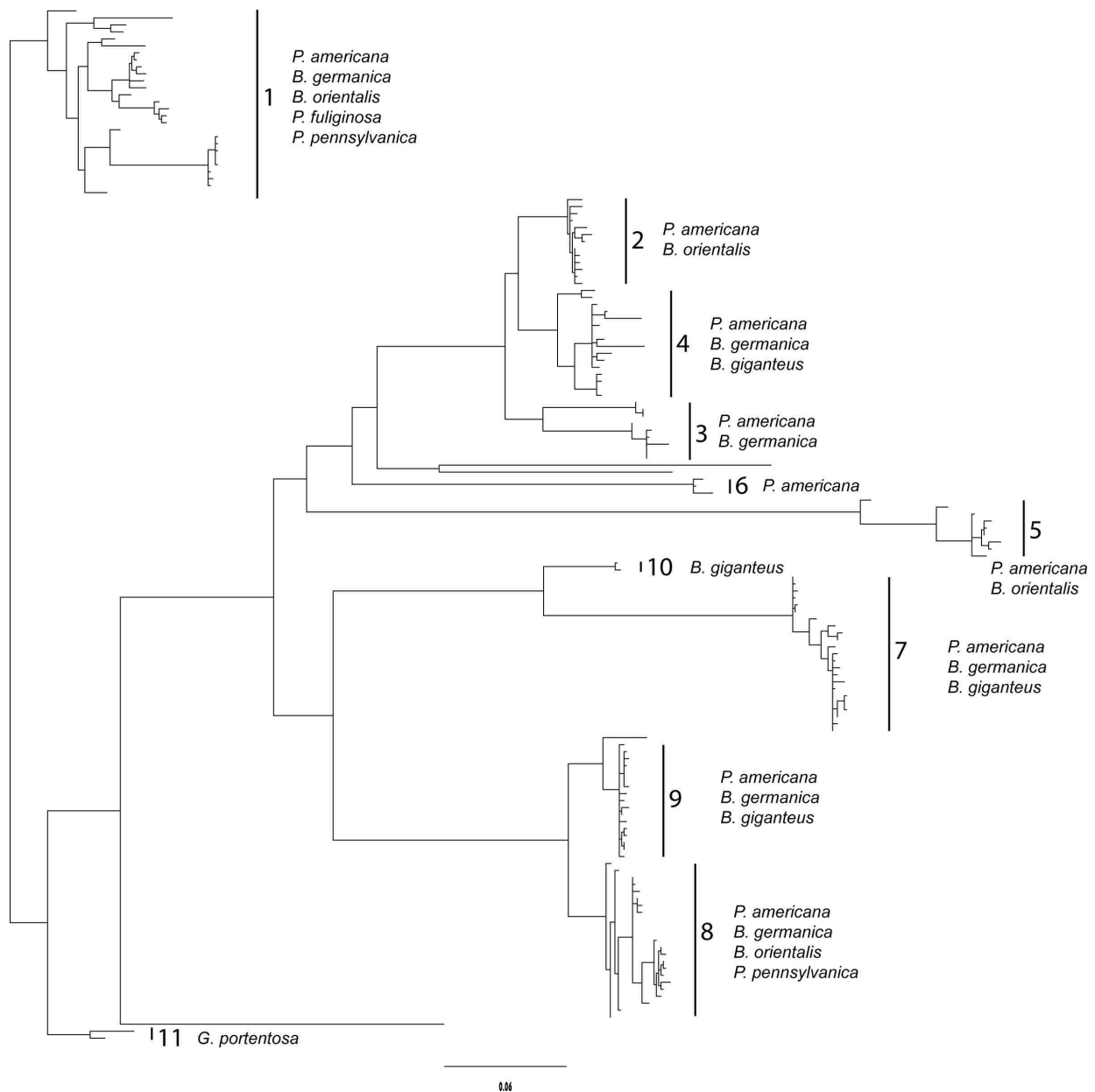


Fig. 8. An unrooted phylogeny of *Entamoeba* SSU rDNA from eight cockroach species: *Periplaneta americana*, *Blattella germanica*, *Blaberus giganteus*, *Gromphadorhina portentosa*, *Blatta orientalis*, *Cryptocercus punctulatus*, *Periplaneta fuliginosa*, and *Parcoblatta pennsylvanica*. Major sub-clades are numbered. Host species with *Entamoeba* in each sub-clade are indicated. Maximum-likelihood with GTR+GAMMA was used to assess relationships between 148 taxa using 1,272 aligned characters.



Fig. 9. An unrooted phylogeny of *Entamoeba* SSU rDNA from 28 cockroaches across eight cockroach species. Major sub-clades are numbered. Host specimens with *Entamoeba* in each sub-clade are indicated. Maximum-likelihood with GTR+GAMMA was used to assess relationships between 148 taxa using 1,272 aligned characters.

Species	Specimen Name	Abbreviation if sequenced	1	2	3	4	5	6	7	8	9	10	11	<i>E. moshkovskii</i>
<i>P. americana</i>	NPA	NPA21, NPA 22												
<i>P. americana</i>	Pa1	Pa1	■			■	■		■	■				
<i>P. americana</i>	Pa2	Pa2	■						■	■	■			
<i>P. americana</i>	Peri 1	Pa1-#or	■		■	■			■	■	■			■
<i>P. americana</i>	Peri 3	Pa3-#or	■		■	■			■	■	■			■
<i>P. americana</i>	Peri 5	Pa5	■					■			■			
<i>P. americana</i>	Peri 6	Pa4		■					■	■				
<i>P. americana</i>	Peri 8	Pa7		■	■									
<i>P. americana</i>	Peri 9	Pa8		■		■								
<i>P. americana</i>	Peri 24	Pa24					■							
<i>P. americana</i>	Peri 32	Pa32	■											
<i>B. germanica</i>	Blat 2	Bge2	■		■	■			■	■	■			
<i>B. giganteus</i>	Blab 1	Bgi1				■			■		■	■		
<i>G. portentosa</i>	Grom 1	Gp1											■	
<i>G. portentosa</i>	Grom 2	Gp2											■	
<i>G. portentosa</i>	Grom 3	Gp1											■	
<i>G. portentosa</i>	Grom 4	Gp1											■	
<i>G. portentosa</i>	Grom 5	Gp1											■	
<i>B. orientalis</i>	Ori 1	Bo1	■											
<i>B. orientalis</i>	Ori 2	Bo2												
<i>B. orientalis</i>	Ori 3	Bo3	■				■			■				
<i>C. punctulatus</i>	Cryp 1	Cp1												
<i>P. fuliginosa</i>	Ful 1	Pf1	■											
<i>P. pennsylvanica</i>	Parc 1	Pp1	■											
<i>P. pennsylvanica</i>	Parc 12	Pp12	■											
<i>P. pennsylvanica</i>	Parc 16	Pp16	■											
<i>P. pennsylvanica</i>	Parc 17	Pp17	■							■				
<i>P. pennsylvanica</i>	Parc 22	Pp22	■							■				

Table 4. Numbered columns indicate clades from Figures 12-14, and *E. moshkovskii* column indicates *E. moshkovskii* clade. Shaded cells indicate that members of the clade were found within the host specimen.

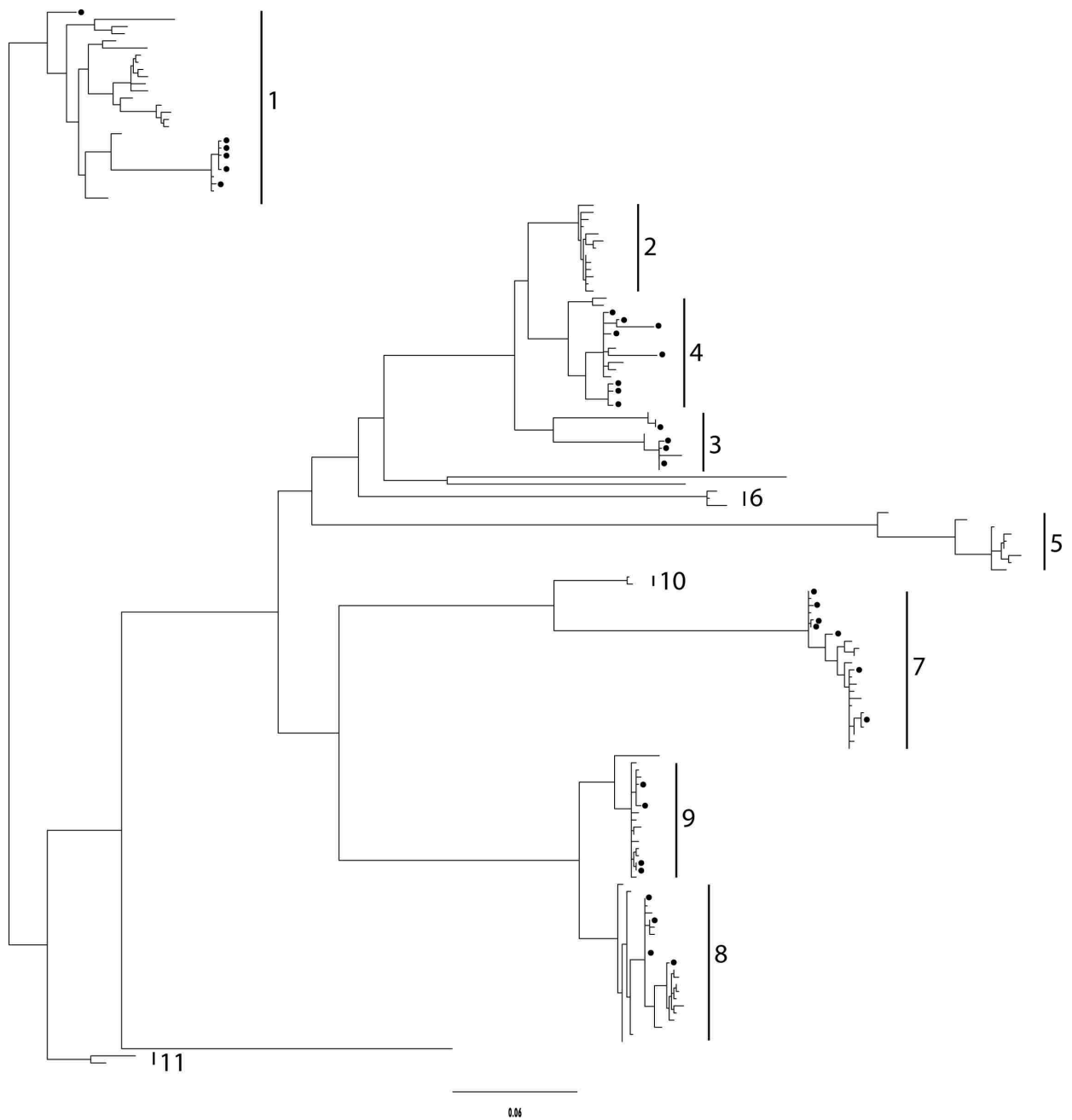


Fig. 10. An unrooted phylogeny of *Entamoeba* SSU rDNA from eight cockroach species. Major sub-clades are numbered. Taxa from non-hindgut regions are indicated with circles. Maximum-likelihood with GTR+GAMMA was used to assess relationships between 148 taxa using 1,272 aligned characters.

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Summary

Molecular evidence supports *Entamoeba* as a well-supported clade in the Archamoeba, a group of amitochondriates within the Amoebozoa. Molecular phylogenetic representations currently contain *Entamoeba* from vertebrates, while ignoring those from non-vertebrates due to a lack of molecular data. *Entamoeba* from non-vertebrates have been characterized only by morphology and a few other non-molecular characters such as host. The taxonomy of vertebrate-inhabiting *Entamoeba* has changed over time, as isolates have been repeatedly lumped and split as new data is collected. SSU rDNA of vertebrate-inhabiting *Entamoeba* has often provided support to pre-existing species delineations, but has also frequently thrown doubt as to whether previously named "species" deserve the distinction. Similarly, SSU rDNA of *Entamoeba* from non-vertebrates could reveal great genetic diversity and be used to test hypotheses of species distinctions.

Cockroaches were chosen as hosts in this study because an initial screening revealed diversity of their *Entamoeba*, they are ecologically important and widespread organisms, and they were a convenient non-vertebrate host to collect. *Entamoeba* SSU rDNA was amplified with the aid of primers designed in our lab to specifically bind to these sequences. A total of 60 cockroaches across eight species was sampled. Thirty-four of these were *Periplaneta americana*. The remaining were *Blattella germanica*, *Blaberus giganteus*, *Blatta orientalis*, *Cryptocercus punctulatus*, *Gromphadorhina portentosa*, *Parcoblatta pennsylvanica*, and *Periplaneta fuliginosa*. This set of species spans 4 of the 6 cockroach families. *Entamoeba* were detected in at least one representative of each host species, showing that *Entamoeba* are found

among phylogenetically distant members of Blattodea, and are likely to be found in additional members of Blattodea. They were detected in all 34 *P. americana* specimens, indicating a high infection rate in the two populations studied- one from captivity, and the other a wild, local one. If all *P. americana* populations have a high infection rate, then cockroach-inhabiting *Entamoeba* could be the most globally common members of the genus!

Sequences were generated from 28 of the 56 cockroaches that tested positive, including at least one cockroach from each species. Multiple variants were found in a single cockroach for many species and for all *P. americana* assessed. Most taxa formed a clade sister to the one predominated by vertebrate-inhabiting *Entamoeba*. This clade contains at least 11 distinct sub-clades. While this indicates great phylogenetic diversity among *Entamoeba* in cockroaches, information on other biologically relevant differences between the clades would be needed before designating new species.

Appendix: Comparison of Stains for Identifying *Entamoeba* in Cockroach Gut

A.1 Introduction

Entamoeba are members of the Amoebozoa that inhabit the guts of many mammals, birds, reptiles, and insects. Morphological studies have focused primarily on *Entamoeba* that are pathogenic in humans and snakes. The morphology and phylogeny of other vertebrate-infecting *Entamoeba* is also being elucidated with the help of molecular techniques (Stensvold et al. 2011). Comparatively little work has been done on insect-infecting *Entamoeba* since their discovery in 1927 (Lucas). Molecular work in our lab has revealed genetic diversity in cockroach-inhabiting *Entamoeba* comparable to that of all other *Entamoeba* combined. However, we still know very little about the morphology of the organisms we are presumably detecting. Some of the difficulties in seeing and identifying *Entamoeba* are: 1) like many colorless amoebae, their outlines are usually invisible under bright field microscopy, 2) darker debris draws more attention than slow moving and transparent amoebae, and 3) distinguishing them from other amoebae can be difficult. Phase contrast is commonly used to overcome the first problem, but it does not add much color to the amoebae, so to the untrained observer they may still be less noticeable than debris.

The nucleus is a prime diagnostic feature for distinguishing *Entamoeba* from other protists. The nucleolar material forms a unique pattern of a central mass surrounded by a peripheral ring. This “bull's eye” pattern is only sometimes visible using phase contrast. If this pattern could be highlighted consistently, it would help in locating *Entamoeba* among debris and distinguishing them from other amoebae. Many stains that bind to nucleolar material also imbue

some coloration to the cytoplasm or cell membrane, so cell shape may also become more visible in the process.

Multiple stains were tested on cultured *Entamoeba* and compared for their ability to highlight nucleolar material. The most optimal stains were then used in roach gut contents to locate *Entamoeba*. Among fluorescent stains, DAPI and Sybr Safe (Invitrogen) were tested. DAPI is often used to visualize nuclei and chromosomes, so it was expected to resolve nuclear details. Sybr Safe is marketed as a stain for nucleic acid in gel electrophoresis, though Biotium (2011) found that it also has the ability to cross cell membranes and stain nuclear DNA. The following non-fluorescent stains were also tested: Wheatley's trichrome, methyl green, Mayer's hematoxylin, iodine, eosin Y, and phloxine B. Wheatley's trichrome and iodine are the most commonly used stains for *Entamoeba*. Eosin Y with phloxine B was used by Tan et al. (2010) with good results for identification. Swierczynski and Milanesi (2010) had good staining of nuclear chromatin in *Entamoeba coli* using Mayer's hematoxylin. There is no record of methyl green being used to stain *Entamoeba*, though it is known to stain nuclei of animal and plant cells dark purple.

A.2 Methods

A.2.1 DAPI and Sybr Safe

A drop of DAPI was added to a drop of PC2, a culture containing *Entamoeba moshkovskii* and bacteria. It was immediately viewed with a Zeiss Axioskop 2 Plus attached to a light source (Zeiss FluoArc) and DAPI filter.

Sybr Safe was mixed with fresh culture in a microfuge tube. After 30 minutes, a drop of the mixture was viewed with a setup similar to above, but with a EGFP filter instead of a DAPI filter. The same protocol was repeated with SAF (sodium acetate formalin)-preserved culture.

A.2.2 Wheatley's Trichrome

The protocol was adapted from Meridian Bioscience. A smear of SAF-preserved culture was dried at room temperature for one to two hours. The slide was placed in 70% ethanol for 10 minutes, a separate jar of 70% ethanol for 5 min., and then another for 5 minutes. It was then placed in trichrome for 8 min., dipped in acid ethanol (100 mL 90% ethanol, .5 mL glacial acetic acid) for 5-10 seconds, dipped twice in 95% ethanol, placed in 95% ethanol for 5 min., a separate jar of 95% ethanol for 5 min., and 100% ethanol for 3 minutes. The slide was removed and viewed when dry.

The following are some of the modifications applied to subsequent trials: xylene for three min. at the end, removal of two 70% ethanol washes, decrease in time of destaining (acid ethanol) dips, increase and decrease of time in trichrome, and fixing with Schaudin's fixative or PVA (polyvinyl alcohol) instead of SAF.

A.2.3 Methyl green

A drop of methyl green was added to a drop of fresh culture and viewed after 5-10 minutes. The stain was diluted to 1:4 and tested with fresh and SAF-preserved culture. To see if incubation time plays a role, fresh culture was mixed with diluted methyl green and mixed in a microfuge tube. A drop was viewed after five minutes, and another after 90.

A.2.4 Mayer's hematoxylin

A drop of Mayer's hematoxylin (2.5 g alum, 0.05 g hematoxylin, 0.01 g sodium iodate, 0.05 g citric acid, 50 mL distilled water) was added to a drop of fresh culture and viewed after five minutes. In the next trial, a drop of culture was smeared on a Poly L lysine-coated slide to encourage retention of cells. It was placed in Schaudin's fixative for 90 minutes, iodine alcohol for 10 minutes, 70% ethanol for 6 minutes, and Mayer's hematoxylin for 5 minutes. The slide was rinsed in running tap water for 10 seconds, distilled water for 10 seconds, placed in xylene for 3 minutes, and viewed when dry.

In the next trial, a weak base (ammonia) was added to make the hematoxylin more apparent. A drop of Mayer's hematoxylin was added to a drop of SAF-preserved culture, and a drop of diluted ammonia was added five minutes later. This was repeated with fresh culture.

A.2.5 Iodine

A drop of freshly made 2% Lugol's iodine (0.2 g potassium iodide, 0.1 g iodine, 10 mL distilled water) was added to a drop of fresh culture and viewed after five minutes. This was repeated with SAF-preserved culture.

A.2.6 Eosin Y, Phloxine B, and Iodine in Various Combinations

A drop of eosin Y + phloxine B (10mg eosin Y, 1mg phloxine B, 7.4 mL ethanol, 390 mL distilled water, 40 mL glacial acetic acid) was added in separate trials to a drop of SAF-

preserved, PVA-preserved, and fresh culture. A drop of $\frac{1}{2}$ x (eosin Y + phloxine B) was added to a drop of SAF-preserved culture.

Eosin Y and phloxine B were then tested individually to determine the contribution of each dye to the staining results, to help in finding the optimal ratio for combining the two. SAF-preserved culture was tested separately with $\frac{1}{8}$ x eosin Y and $\frac{1}{2}$ x phloxine B. After testing each stain individually, the following ratios of eosin Y: phloxine B were mixed from stock solutions (eosin Y stock: 0.1 g eosin Y, 10 mL distilled water; phloxine B stock: 0.1 g phloxine B, 10 mL distilled water) and tested on fresh culture: 4:1, 3:2, 2:3, and 1:4.

Eosin Y + phloxine B was mixed with iodine to see if this would increase the contrast between cellular components. Ratios of 9:1 and 8:2 of (eosin Y + phloxine B): iodine were tested.

A.2.7 Roach Gut

The gut of a juvenile giant cockroach (*Blaberus giganteus*), raised from adults from Carolina Biological Supply Company, was used for *in situ* examination. The roach was euthanized by exposure to ethyl acetate in a sealed container, followed by evisceration. Portions of the hindgut were placed in various buffers and mixed with a pipette. A drop of this solution was viewed on a slide with no stain, eosin Y + phloxine B (3:2), or iodine + eosin Y + phloxine B.

A.3 Results

A.3.1 DAPI and Sybr Safe

After DAPI was added to fresh culture, nuclei were visible and could be counted in some cysts (Fig. 1 b-d). However, intra-nuclear details could not be resolved.

Bacteria glowed green after Sybr Safe was added to fresh culture, but *Entamoeba* cells did not fluoresce. Trophozoites were still active 30 minutes after adding the stain. When Sybr Safe was added to SAF-preserved culture and incubated for 30 minutes, nuclei could be seen and counted in some cysts. However, in many cases the nuclei were so bright that they could not be resolved from each other (Fig. 2).

A.3.2 Wheatley's Trichrome

In the first trial, cysts were blurry and unstained. Cysts were colored in later trials, but often rugged on the surface and stained homogeneously, rendering internal details indiscernible. Nuclei were sometimes visibly stained in PVA- and SAF-fixed samples, but their details were not discernible.

A.3.3 Methyl Green

Adding undiluted stain to fresh culture resulted in an intensely darkened field. When it was added to SAF-preserved culture, internal details of cysts appeared distorted. Addition of diluted stain to fresh culture stained approximately one of every 40 cysts. A bullseye nucleus was visible in stained cysts (Fig. 3). Some trophozoites may have also been stained. Adding

diluted stain to SAF-preserved culture stained some cysts slightly, but nuclei were not discernible. Incubation of diluted stain with fresh culture for 5-90 minutes did not result in nuclear staining.

A.3.4 Mayer's Hematoxylin

Adding Mayer's hematoxylin to fresh culture did not stain cells, and adding ammonia water did not improve results. Adding Mayer's hematoxylin to SAF-preserved culture, followed by ammonia water, did not stain cells. Fixing in Schaudin's and running in tap water resulted in a few cysts becoming dark brown, but no internal structures were discernible.

A.3.5 Iodine

Adding iodine to fresh culture imbued an amber color to the cytoplasm of all cysts and trophozoites. Nuclei and nuclear structure were visible in some cells. Iodine reacted when added to SAF-preserved culture, and cells were no longer identifiable.

A.3.6 Eosin Y, Phloxine B, and Iodine in Various Combinations

Adding eosin Y + phloxine B to SAF-preserved culture increased the visibility of trophozoite morphology and nuclear detail. Cysts were also stained, but their nuclei were not discernible. Adding the stain to PVA-preserved culture also increased visibility of cyst and trophozoites, though the trophozoites appeared slightly rugged. Nuclei were sometimes visible in trophozoites, though not as often or as clearly as in SAF-preserved stained specimens. Nuclei were sometimes very slightly visible in cysts. Adding eosin Y + phloxine B to fresh culture

stained some cysts, but many remained unstained, even after 30 minutes on the slide.

Trophozoite nuclei were sometimes slightly stained, but not as much as in SAF-preserved stained specimens.

Adding 1/8 x eosin Y to SAF-preserved culture resulted in light pink cells. In trophozoites, nuclei became slightly more visible. For cysts, internal details and nuclei were sometimes slightly more discernible than in unstained culture. Adding 1/2 x phloxine B to SAF-preserved culture made nuclei more visible in trophozoites. Two nuclei were observed in some cells, but it was unclear whether these were rounded pre-mitotic trophozoites or cysts. Iodine + eosin Y + phloxine B differentiated cysts from trophozoites when added to fresh culture. Trophozoites were orange-brown to pink, and cysts were yellow. Trophozoite nuclei and nuclear structure were visible in all cells, and cyst nuclei were slightly visible in a few cases.

Different ratios of eosin Y to phloxine B were tested to find the optimal ratio. A ratio of 4:1 added to fresh culture resulted in some cells staining only faintly, with nuclei visible in some trophozoites. With the 3:2 stain, nuclei were visible in many trophozoites, and could even be counted in some cysts. With the 2:3 stain, cells were very dark purple. Though trophozoite nuclei were still visible, the cells were too dark to see interior details as clearly as with 4:1 or 3:2 stains. The 1:4 stain increased visibility of cyst nuclei. Of the ratios, 3:2 gave the most optimal results, so the 3:2 stain was also tested on PVA-preserved culture. The stain and culture reacted when combined. The field appeared dull pink and cells did not stain differently from the background.

Eosin Y and phloxine B were added to iodine in different ratios to see if contrast between cellular components could be improved. Using a 9:1 ratio of eosin Y + phloxine B to iodine

resulted in little difference compared to only eosin Y + phloxine B. An 8:2 ratio resulted in differentiation between cysts and trophozoites, as cysts appeared yellow and trophozoites pink. Some pink cells were round, but these were believed to be rounded trophozoites rather than cysts.

A.3.7 Roach Gut

A variety of amoebae were visible in both unstained and stained culture. Several spherical, cyst-like objects were seen with faintly visible spheres inside (Fig. 4). Visibility of these internal spheres was significantly enhanced by both stains tested on roach gut (eosin Y + phloxine B (3:2) and iodine + eosin Y + phloxine B). These stains darkened the edges and center of the spheres to reveal a bullseye pattern. When the stain mixture that contained iodine was used, some debris was stained yellow, but no yellow-stained cysts were visible as in trials on the cultured cells.

Many amoebae were stained, with morphology and internal structures visible. Several spheres were seen with 1, 2, or 3 bullseye structures inside. Bullseye structures were not visible in any highly amoeboid-shaped cells.

A.4 Discussion

Eight stains and stain combinations were compared for their ability to stain *Entamoeba* nuclei, and the two best were chosen for examination of cockroach gut: eosin Y + phloxine B (3:2), and iodine + eosin Y + phloxine B. Both can be used with fresh and SAF-preserved culture.

The fluorescent stain DAPI caused nuclei to fluoresce, but nuclear details were not visible. The inability of Sybr Safe to stain nuclei in fresh culture is likely due to lack of penetration of live cells. Wheatley's trichrome was the first non-fluorescent stain tested because it was expected to give the best results. This staining procedure is widely used in the medical field for detecting *Entamoeba histolytica* in fecal specimens. Of the stains tested, the greatest time and effort was spent troubleshooting Wheatley's trichrome because of its good reputation. However, the cells became distorted and/or unstained in every trial with this stain. Methyl green produced beautiful looking cysts with good contrast between nuclei and cytoplasm. However, it only stained a small portion (1/40) of cysts and no trophozoites, so is not ideal for identifying *Entamoeba in situ* where the population may be low. Mayer's hematoxylin has been used by others to darken the “bullseye” of *Entamoeba* (Swierczynski Milanese 2010), but here it did not appear to penetrate cells. Staining with iodine was quick and easy, and sufficiently improved the visibility of nuclei in some cells. Eosin Y + phloxine B stained cytoplasm and nuclei of trophozoites and cysts. The ratio of the two dyes was adjusted to provide optimal nucleus-to-cytoplasm contrast.

Iodine is commonly used to visualize internal details of *Entamoeba*, though Tan et al. (2010) found it inferior to Wheatley's trichrome and eosin Y + phloxine B. Our results confirm that eosin Y + phloxine B was more effective than iodine. Eosin Y + phloxine B seems to be a very practical stain for *Entamoeba* detection, as it is much easier and faster to use than Wheatley's, and more effective than iodine.

Adding iodine to eosin Y + phloxine B allowed for differentiation of cysts and trophozoites, as well as greater contrast between debris. This may be particularly helpful for

discriminating between rounded-up trophozoites and cysts, which are both spherical in shape. This is the first described use of iodine + eosin Y + phloxine B as a stain for *Entamoeba*, and possibly for any cell.

Eosin Y + phloxine B (3:2) and iodine + eosin Y + phloxine B were used for staining squashed gut from juvenile *B. giganteus*. They both stained amoebae and increased the visibility of interior structures. The iodine did not provide any benefit *in situ*, and made the field appear slightly darker. Therefore, eosin Y + phloxine B (3:2) appears to be the optimal stain for *in situ* use. Cysts (Fig.4 c,d) and rounded-up amoebae (Fig.4 b) containing bullseye nuclei were observed. The next major goal is to further confirm the identity of these potential *Entamoeba* and link genetic variants to particular morphologies. Continued sampling with the staining methods refined here, as well as application of new techniques such as fluorescence *in situ* hybridization, could prove invaluable in moving towards this goal.

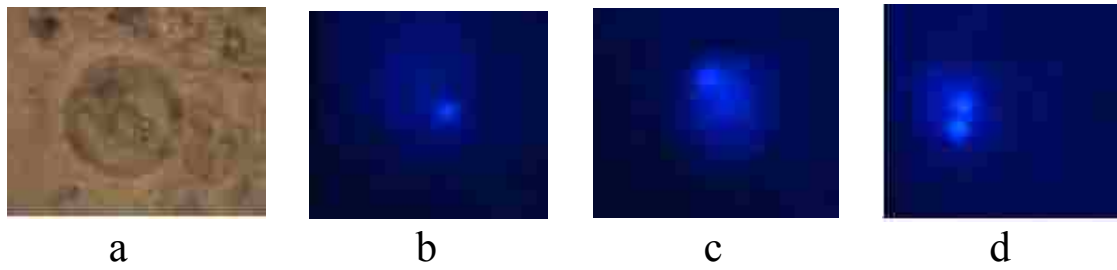


Fig. 1. *Entamoeba* cyst from culture PC2 a) unstained, 400x magnification. b-d) with DAPI, 400x mag. Four nuclei visible when viewing through specimen.

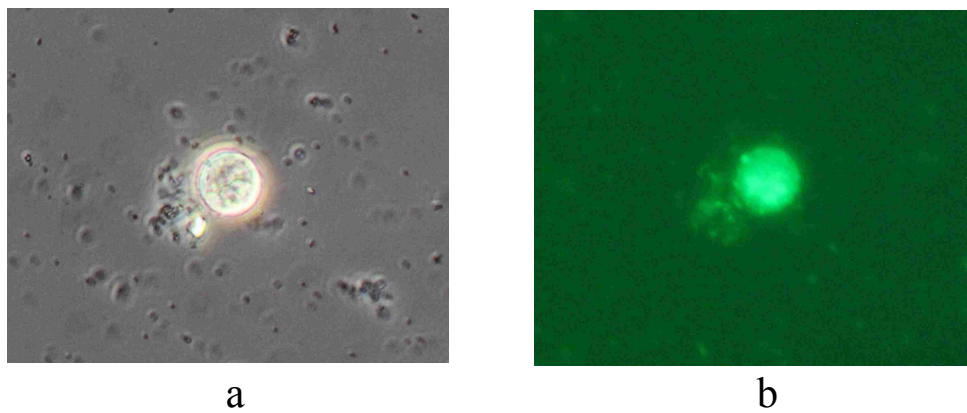


Fig. 2. *Entamoeba* cyst from culture PC2 a) unstained, 400x mag. b) after 30 minute incubation with Sybr Safe, 400x mag.



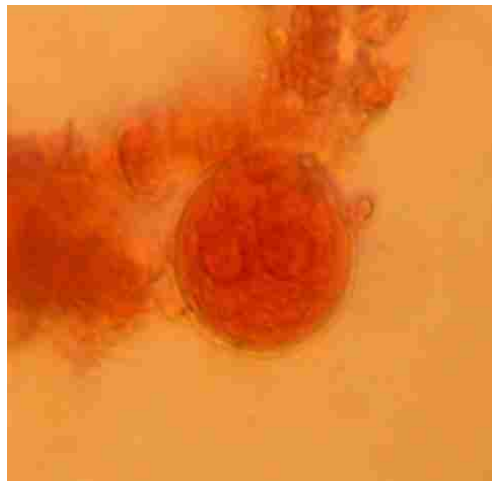
Fig. 3. *Entamoeba* cyst from culture PC2, incubated with methyl green for 10 minutes.



a



b



c



d

Fig. 4. Cysts and trophozoites from *B. giganteus* gut, possibly *Entamoeba*. a) cyst, unstained, 400x mag. b) rounded trophozoite with bullseye nucleus, eosin Y + phloxine B (3:2), 400x mag. c) cyst with at least 2 nuclei, eosin Y + phloxine B (3:2), 400x mag. d) cyst or rounded trophozoite with bullseye nucleus, eosin Y + phloxine B (3:2), 1000x mag.

A.5 Works Cited

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