Tritrichomonas foetus: Search for a Potential Reservoir Host in Wild and Captive Rodents

by

Isaac Harrison McNeely

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Approved by

Christine A. Sundermann, Chair, Professor of Biological Sciences
Dr. Robert S. Lishak, Associate Professor of Biological Sciences
Dr. Troy L. Best, Professor of Biological Sciences
Dr. Mark R. Liles, Associate Professor of Biological Sciences

Abstract

Tritrichomonas foetus is a parasitic protist that has economic importance because it causes a venereal disease in cattle and a bowel disease in domestic cats. The source of infection for feline populations is unknown; one possibility is that the parasite is carried by rodents, which often contain similar protists in the cecum.

In this study, samples of cecal material were taken from 75 wild and 51 domestic rodents in Alabama and Georgia. Samples were examined microscopically for the presence of trichomonads, especially those large enough to be *T. foetus* (>15 μm long). These large trichomonads were found in 59.5% of samples. To determine the species of trichomonads present, PCR and sequencing methods were used. Three sets of primers were used to amplify the conserved 5.8S rRNA gene, as well as the flanking regions ITS-1 and ITS-2. Results of PCR indicated that *T. foetus* was present in 28.3% of all specimens. Products derived from PCR were sequenced, and compared to the GenBank database was used to determine sequence homology. Of the specimens, 14.3% were identified as *T. foetus* (n = 18 of 126), and 12.7% were identified as *T. muris* (n = 16 of 126). This indicates that rodents could be a reservoir host for *T. foetus*.

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Table of Contents

List of Tables

List of Figures

CHAPTER I: INTRODUCTION AND REVIEW OF LITERATURE

Tritrichomonas foetus is an anaerobic parasite within the taxonomic groups Excavata: Parabasalia: Trichomonadida (Adl et al., 2005), although the precise hierarchy of classification of protists is still debated (Cavalier-Smith, 2003; Adl et al., 2005; 2007). Protists of subgroup Trichomonadida have a tube-like axostyle that runs the length of the pear-shaped cell body and continues past the posterior end of the cell. They also have 3 to 5 flagella on the anterior end and 1 recurrent flagellum that runs toward the posterior end of the cell. The recurrent flagellum supports an undulating membrane down the length of the cell body. Internally, trichomonads have an oval or elongate nucleus as well as a parabasal body near the anterior end of the cell. (Honigberg, 1963). Trichomonads also have hydrogenosomes, a peculiar type of organelle in which ATP is generated under anaerobic conditions via oxidation of pyruvate, and molecular hydrogen is generated as a waste product (Lindmark and Müller, 1973; Müller, 1993). Presence of hydrogenosomes instead of mitochondria was once believed to indicate that trichomonads are evolutionarily primitive species. However, trichomonad hydrogenosomes have 2 membranes, indicating a history as an endosymbiont similar to mitochondria (Benchimol and De Souza, 1983). Further evidence supports the hypothesis that hydrogenosomes are derived from mitochondria, including the presence of mitochondrial DNA in the nucleus of trichomonads and a complex method of protein transport similar to what occurs in mitochondria (Embley et al., 2003; Dyall et al., 2004; Gray, 2005).

Tritrichomonas foetus is a member of the phylogenetic group Tritrichomonadinae, originally a subfamily of Trichomonadidae. Recent studies have identified this group as a separate class, Tritrichomonadea (Figure 1.1; Cepicka et al., 2010). The tritrichomonads have an axostyle that is larger in diameter than that of trichomonads, with a relatively slender costa, and a

comb structure that may replace the costal base of other trichomonads (Honigberg, 1963; Honigberg et al., 1971). They also have a characteristic locomotion that includes rotational movement of the cell as well as jerking movements of the flagella (Wenrich and Emmerson, 1933).

Trophozoites, the active feeding stage of *T. foetus*, are generally 10 to 25µm in length and usually one-third to two-fifths as wide as they are long. The recurrent flagellum and undulating membrane (3 and 4 in Figure 1.2) run posteriorly down the dorsal surface of the cell and have 4 to 5 transient undulations at any given time. The recurrent flagellum also runs past the end of the undulating membrane at the posterior end of the cell (6 in Figure 1.2). The axostyle (8 in Figure 1.2) has a capitulum at the anterior end that contains several endoaxostylar granules (9 in Figure 1.2). At the posterior end, the axostyle emerges from the cell and quickly tapers to a conical spine (Figure 1.3). The nucleus (14 in Figure 1.2) is oval, is 3.5 to 5µm long, and is generally in the anterior one-third of the cell (Wenrich and Emmerson, 1933). The parabasal body is 3 to 4 µm long and is dorsal and to the right of the nucleus (12 in Figure 1.2; Mattos et al., 1997).

Tritrichomonas foetus obtains nutrients by phagocytosis and can break down other cells for nutrition. The parasite adheres to host cells by connecting to laminin proteins in the basal membrane (Silva-Filho et al., 1988; Petrópolis et al., 2008). It does this by first using the posterior flagellum to attach and then adhering to the cell body (Corbeil et al., 1989). When the trophozoite has adhered, it may change to a more amoeboid form to perform phagocytosis (De Carli et al., 2004; Singh et al., 2004). *Tritrichomonas foetus* also releases a cysteine proteinase (CP8) that can break down a variety of proteins (Talbot et al., 1991). This proteinase

may be host-specific because certain amino acids can allow or prevent binding (Lucas et al., 2008). CP8 may also cause cellular apoptosis as was demonstrated in bovine vaginal epithelial cells (Singh et al., 2004).

There are 3 species of trichomonads in humans: *Trichomonas tenax, Pentatrichomonas hominis,* and *Trichomonas vaginalis*. *Trichomonas tenax* is a commensal of the mouth, and *P. hominis* is symbiotic in the colon. *Trichomonas vaginalis* causes the common venereal disease, trichomoniasis. Infected males often are asymptomatic, and the majority of symptoms of trichomoniasis are seen in the female reproductive tract in the form of inflammation, itching, and malodorous discharge. In pregnant women, trichomoniasis may also cause premature birth, low birth weight, or both (Workowski and Berman, 2010).

Tritrichomonas foetus has an effect similar to *T. vaginalis* in cattle (*Bos taurus*), but the disease is more drastic in that it can cause spontaneous abortion of a fetus and possible sterility of the infected female (Morgan, 1947). The venereal disease caused by *T. foetus* in cattle is bovine trichomoniasis. The protist usually occurs in the preputial cavity of bulls and in epithelial crypts on the penis (Bondurant, 1985). At the time of infection, trophozoites of *T. foetus* may cause swelling and inflammation of the preputial tissue, but symptoms usually disappear within 2 weeks after which the bull is usually an asymptomatic carrier (Honigberg, 1978; Felleisen, 1999). Compared to older bulls, younger bulls are less likely to become permanent carriers of the parasite, but they are still capable of spreading the parasite to females (Da Silva et al., 2011). Transmission of *T. foetus* to the cow occurs during breeding; as the parasite spreads into the uterus and oviducts, an inflammatory response occurs that may prevent fertilization or kill a developing embryo. Abortions usually occur 1 to 16 weeks after breeding (Morgan, 1947; Bondurant, 1985). The fetus and placenta often will have lesions that can be used to diagnose

trichomoniasis after the abortion (Rhyan et al., 1988). If all fetal membranes are not expelled after abortion, the cow may develop chronic endometritis and pyometra, which can cause permanent sterility (Wenrich and Emmerson, 1933). Infected cows usually will mount an immune response that can clear the infection in 2 to 4 months; however, there is no reliable cure for infected bulls, which often are culled. The chance of infection can be reduced by artificial insemination and use of virgin bulls for breeding, but these measures can be very costly (Rae, 1989). However, artificial insemination does not completely prevent infection by *T. foetus* because the parasite can survive freezing in semen ampules (BonDurant, 1985).

Tritrichomonas foetus has been found in other species of hosts in addition to cattle. *Tritrichomonas suis*, a commensal of nasal passages and gastrointestinal tracts of swine (*Sus scrofa*), has been suggested to be the same species as *T. foetus* due to morphological and genetic similarities (Mattos et al., 1997; Tachezy et al., 2002; Lun et al., 2005). Recent genetic data have also indicated that *Tritrichomonas mobilensis*, a parasite of the intestinal tract of squirrel monkeys (*Saimiri sciureus*), may be the same species as well (Kleina et al., 2004). *Tritrichomonas foetus* has also occasionally been found in dogs (*Canis lupus familiaris*) with diarrhea, sometimes alongside *P. hominis*, but its pathogenicity in dogs is unknown (Tolbert et al., 2012).

Tritrichomonas foetus also has been found in domestic cats (*Felis cattus*) where it causes bowel infections and chronic diarrhea (Levy et al., 2003). The original source of this infection is unknown, but transmission appears to occur between cats via the fecal-oral route, and the disease occurs most often at densely populated catteries (Foster et al., 2004). Infected felines can be asymptomatic or have diarrhea with blood, mucus, a foul odor, or a combination of these. Other symptoms include flatulence, tenesmus, and incontinence (Manning, 2010). These symptoms

may wane over time in a cat that is still infected. Most cats are diagnosed at ages <1 year with no difference in susceptibility between males and females (Gookin et al., 1999). Symptoms last from 2 days to 3 years, but most cases resolve themselves within 2 years (Foster et al., 2004). *Tritrichomonas foetus* was recognized initially via light microscopy and PCR testing of fecal specimens of felines (Levy et al., 2003) and is now more commonly detected using the commercially available InPouch TF culture system that originally was created for detection of *T. foetus* in cattle. Testing of this system against cultures of other intestinal parasites of felines such as *Giardia lamblia* and *P. hominis* showed that the culture system was specific enough to culture only *T. foetus* (Gookin et al., 2003).

Recently there has been some question of whether isolates of *T. foetus* in feline and bovine hosts are actually the same species. In a pair of experiments by Stockdale et al. (2007, 2008), attempts were made to cross-infect clean cows with a feline isolate of *T. foetus* and to infect cats with the bovine isolate. In the experiment using cattle, 8 heifers were inoculated with the feline isolate of *T. foetus*. Five of them were still culture-positive after 20 weeks, the normal amount of time for an immune response to develop to fight off an infection by the bovine isolate. Three of the heifers were culture-positive after 30 weeks. While this is within the known range of a persistent bovine infection with *T. foetus*, it indicates the possibility of a phenotypic difference in the feline isolate (Stockdale et al., 2007). In the feline-host experiment, 6 cats were inoculated with the bovine isolate of *T. foetus*. Of these, only 1 was culture-positive through fecal samples at 32 days post-inoculation. Cats were sacrificed 5 weeks post-inoculation and underwent necropsy. The culture-positive animal and 1 other had *T. foetus* trophozoites in the cecum. The differing rates of infection suggest differences in biology or pathogenic behavior of the 2 isolates of *T. foetus* (Stockdale et al., 2008). However, differences in phenotypic behavior

do not necessarily indicate a difference in species. Additionally, the parasites used in these experiments were not cloned before use, which leaves some doubt of their specific strain.

A genetic comparison of feline isolates and bovine isolates of *T. foetus* found only 1 polymorphism in the ITS1-ITS2 region around the gene for 5.8S ribosomal RNA, and that polymorphism was consistent across multiple isolates (Šlapeta et al., 2010). The authors of the study concluded that the 2 isolates were not separate species but were separate genotypes. They also suspected that the separate genotypes may be indicative of differences in parasite host specificity, although the genotypes may not be exclusive at this point. Additional studies found differences in the 2 isolates at the CP8 gene, which may be involved in host specificity (Sun et al., 2012), as well as a semi-conserved portion of the elongation factor 1 alpha gene (Reinmann et al., 2012). The latter study also suggested a difference between the bovine isolate of *T. foetus* and synonymous species *T. suis.*

Because *T. foetus* can be present in such diverse hosts, the question arises as to how it might be transmitted. *Tritrichomonas foetus* has a simple life cycle that consists almost entirely of the trophozoite (active feeding) stage that reproduces by longitudinal fission (Honigberg, 1963). The only notable morphological change that *T. foetus* may undergo is the formation of a pseudocyst, which is a spherical, nonflagellated form that offers some protection when an environment becomes inhospitable due to changes such as pH or temperature. In this form, the flagella are withdrawn into vacuoles and the axostyle and costa change to a curved shape (Pereira-Neves et al., 2003). The organism can still undergo mitosis in this form but will not undergo cytoplasmic division until its environment is more suitable. This event resembles budding of new trophozoites from the pseudocyst (Pereira-Neves and Benchimol, 2009). The pseudocyst may also exhibit increased behaviors of cellular adhesion and the release of

cytotoxins in the presence of host cells (Pereira-Neves et al., 2012). Due to its lack of a true cell wall the pseudocyst is susceptible to heat and desiccation, which makes it unable to survive for long periods outside of a host. Pseudocysts can survive for a short time in material that provides moisture, such as feline urine or wet cat food, but will quickly die in water (Rosypal et al., 2012). The longest recorded survival of *T. foetus* outside of a host is 7 days in a moist fecal sample at room temperature (Hale et al., 2009). Obviously, a pseudocyst cannot persist for lengthy periods outside the host when compared to the longevity of a true cyst or egg. Because the pseudocyst cannot persist outside the host environment for long, it may require a vector or reservoir host. Experimental data have shown that *T. foetus* can survive passage through the alimentary tract of slugs that feed on infected cat feces. In that study, the slugs consumed food that had been seeded with 10^6 trichomonads. The slugs shed infected feces for 1 to 2 days (Van der Saag et al., 2011), which demonstrated that slugs might spread *T. foetus* to some degree but do not act as a long-term reservoir.

One idea that my thesis explores is the potential for rodents to harbor *T. foetus*. For example, one possible reservoir host is the hispid cotton rat, *Sigmodon hispidus*. These rats are native to Neotropical North and South America ranging from the Atlantic coast of the United States westward to Arizona and New Mexico and from southern Nebraska to the northern coast of South America (Cameron and Spencer, 1981). These rodents occupy open field habitats with tall grasses, which supply them with overhead cover as well as food (Fleharty and Olson, 1969; Cameron and Spencer, 1981). Thus, hispid cotton rats are likely to be in cattle-grazing habitats. They are active during both day and night (Cameron and Spencer, 1981) and due to their large populations are likely to be a common prey for cats. This implies that if *T. foetus* is carried by

the hispid cotton rat or any other prey rodent, it could have direct access to the digestive tract of feline hosts.

Many species of rodents have cecal trichomonads, many of which are morphologically similar to *T. foetus* (Boggild et al., 1996; Baker, 2007). These flagellates can represent a variety of species (Table 1.1) including *P. hominis* and *Tritrichomonas muris* (Baker, 2007). At least 1 flagellated species in *S. hispidus* is not yet described (Boggild et al., 2002). Identification of microbes in the cecum can be difficult, especially in wild rodents where a variety of species may be present concurrently. Fortunately, pathogenicity of *T. foetus* in cattle has led to development of several diagnostic methods of detection. The most decisive of these is a polymerase chain reaction (PCR) using primers that specifically target a conserved region of the genome. Felleisen (1997) designed a set of primers to target the area between the genes for 18S and 28S subunits of ribosomal RNA in trichomonads. This included the 5.8S subunit, as well as internal transcribed spacer (ITS) regions 1 and 2, a region of about 372 base pairs (bp) in tritrichomonads. Using these primers he was able to sequence the DNA of several isolates of trichomonads and identify differences in sequences between related species. He was able to demonstrate that the ITS regions were more variable between closely related species than the 5.8S region (Felleisen, 1997). From these data he also was able to design a set of primers known as TFR3 and TFR4 that would anneal specifically to the ITS regions of *T. foetus* (Felleisen et al., 1998). This method was later improved by the addition of another set of primers that could be nested within the range of TFR3 and TFR4. Primers TFITS-F and TFITS-R were designed to focus on the ITS region 1 and part of the 5.8S region and resulted in a smaller (208bp) PCR product (Gookin et al., 2002). It was determined that the use of both primers in a single-tube-nested PCR was more sensitive to the presence of *T. foetus* in fecal samples than either of the

primer sets on their own (Gookin et al., 2002). This combination of primers should be able to identify *T. foetus* in cecal contents from rodents, even in the presence of related species. If *T. foetus* is present in rodents, it could help determine whether feral reservoirs of the parasite exist.

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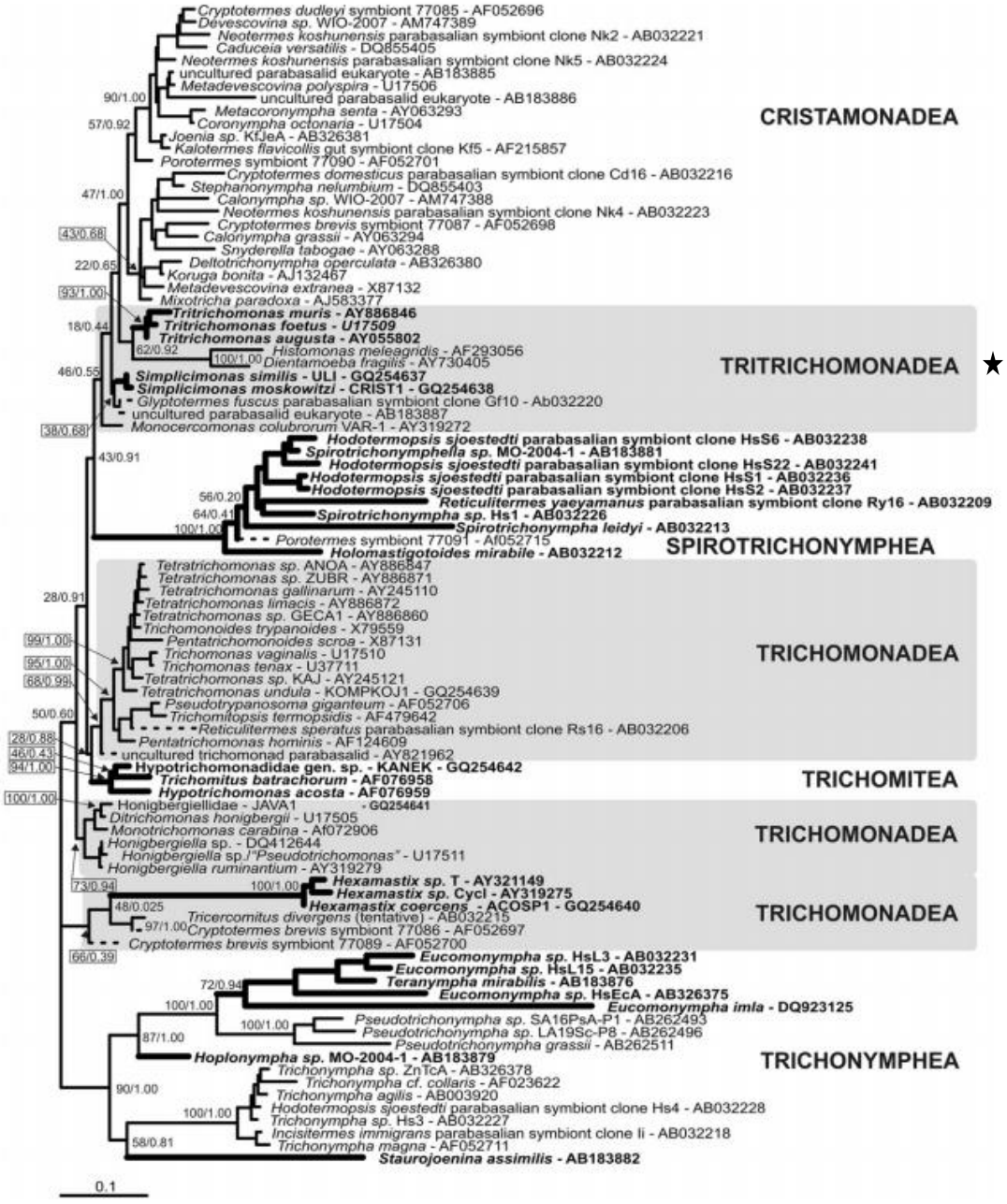

Figure 1.1. Phylogenetic tree of parabasalids based on small subunit ribosomal RNA. Shaded areas indicate division into 6 classes with *Tritrichomonas foetus* and related species placed in Tritrichomonadea (black star). Bold lines indicate family-level relations; dashed lines indicate unsorted parabasalids (Cepicka et al., 2010).

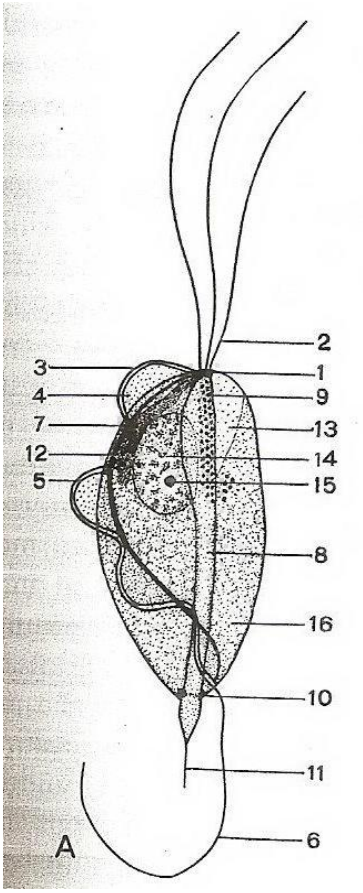

Figure 1.2. Drawing of *Tritrichomonas foetus* with organelles (Wenrich and Emerson 1933). Labelled organelles and structures are numbered as follows: 1) blepharoblast; 2) anterior flagella; 3) posterior flagellum; 4)undulating membrane; 5)accessory filament in undulating membrane; 6) posterior flagellum; 7) costa; 8) axostyle; 9) endostylar granules; 10) chromatic ring; 11) terminal spine of axostyle; 12) parabasal body; 13) cytostome;
14) nucleus; 15) caryosome; 16) cytoplasm.

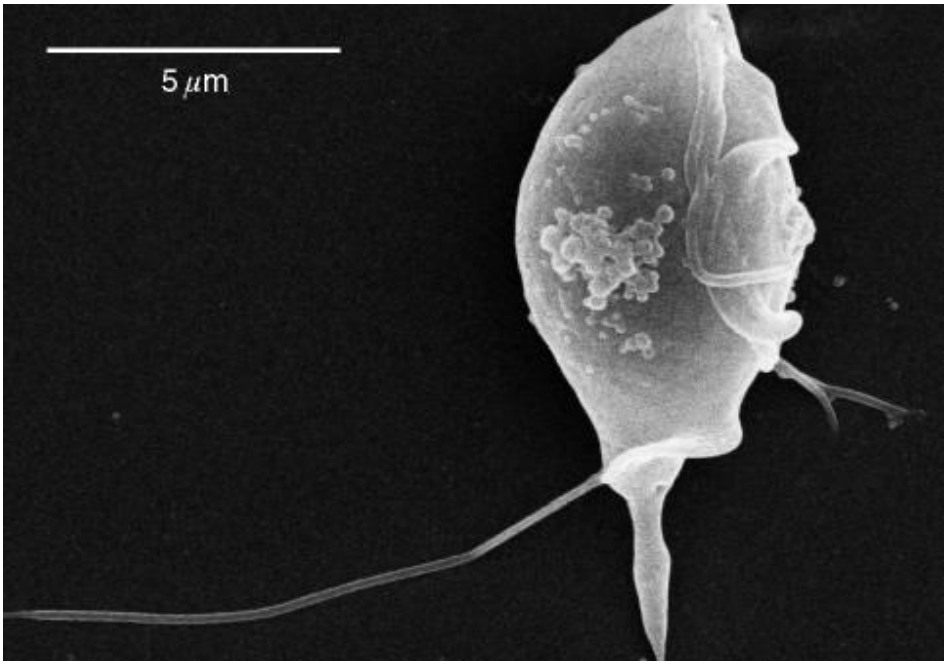

Figure 1.3. Scanning electron micrograph of *Tritrichomonas foetus* (Lim et al. 2010). Note the undulating membrane on the right and emerging axostyle at the bottom of the cell.

Species	Length (µm)	Host
Chilomastix bettencourti	8 – 21	Cecum of mouse, rat, hamster
Giardia muris	7 – 13	Small intestine of mouse, rat, hamster
Hexamita muris	7 – 9	Cecum of mouse, rat, hamster, wild rodents
Monocercomonoides	5 – 15	Cecum of rat, hamster
Octomitus pulcher	6 – 10	Cecum of mouse, rat, hamster, ground squirrel
Pentatrichomonas hominis	9 – 20	Cecum of mouse, rat, hamster, cat, dog, cattle, primates including humans
Retortamonas	4 – 9	Cecum of rat
Spironucleus muris	7 – 9	Cecum of mouse, rat, hamster, wild rodents
Tetratrichomonas microti	4 – 9	Cecum of mouse, rat, vole, other rodents
Trichomitus wenyoni	4 – 16	Cecum of house mouse, brown rat, hamster
Tritrichomonas foetus	10 – 15	Genitalia of cattle; cecum of swine, domestic cat
Tritrichomonas minuta	4 – 9	Cecum of mouse, rat, hamster
Tritrichomonas muris	16 – 26	Cecum of mouse, rat, hamster, gerbil

Table 1.1. Flagellated protists similar in size or shape to those mentioned by Boggild et al. (1996) and their hosts (Baker 2007; Levine, 1961, 1985).

CHAPTER II: SURVEY OF CECAL FLAGELLATES IN RODENTS FROM ALABAMA

Rodents carry a variety of parasites and diseases, which can be spread to humans or other animals (Meerburg et al., 2009). These can include viruses (Mills et al. 1999), protists, and intestinal helminths (Harkema, 1936; Sogayar and Yoshida, 1995; De Lima et al., 2002).

Flagellated protists are commonly in the cecum of rodents where they often reside as commensals instead of parasites. Common cecal flagellates include *Giardia muris*, *Giardia microti*, *Chilomastix bettencourti*, *Pentatrichomonas hominis*, *Tritrichomonas minuta*, and *Tritrichomonas muris* (Doran, 1954; Baker, 2007). These protists are spread through fecal contamination of food and water or through deliberate coprophagy (Saxe, 1954). In some instances there may also be a chance for transmission of protists through cannibalism, as rodents typically ingest the viscera first and would thus receive a large inoculum of contents from the alimentary canal (Hegner, 1936; Saxe, 1954).

Some species of rodents are more likely than others to carry cecal flagellates. For example, Doran (1954) catalogued studies that found 11 species of flagellates in the brown rat, *Rattus norvegicus*. In his catalogue, he cited studies that found only 5 flagellated species in the white-footed deermouse *Peromyscus leucopus* and none in the hispid cotton rat *Sigmodon hispidus*. The cotton rat was not being ignored in surveys of parasites, as many papers described trypanosomes, cestodes and nematodes in this species (Doran, 1954; 1955). It was not until 1996 that a cecal flagellate was reported in *S. hispidus*, and it was reported as an unidentified flagellate of *Tritrichomonas* (Boggild et al., 1996). The reported trichomonad varied from 15 to 24 µm in length and 9 to 12 µm in width, which places it within the size ranges of both *T. muris* and *Tritrichomonas foetus*. (Table 2.1; Levine, 1961). Because this trichomonad was discovered in a

captive colony of *S. hispidus*, it is not known whether the flagellate is common in wild populations.

Tritrichomonas muris has been found in a variety of rodents including *Neotoma fuscipes*, *Peromyscus maniculatus*, and *P. leucopus*, as well as in both wild and captive populations of *R. norvegicus* and *Mus musculus* (Harkema, 1936; Doran, 1954) and is considered common in laboratory populations. Due to this fact and its lack of pathology in rodents, *T. muris* is rarely reported in surveys for parasites of rodents (Baker, 2007). Studies of the fine structure of tritrichomonads have determined that *T. muris* and a few related species may represent a subtype of *Tritrichomonas* that differs from species such as *Tritrichomonas augusta* and *T. foetus* (Honigberg, 1963; Wendell et al., 1971). Part of this distinction is that trichomonads of the *T. muris* type cannot be isolated in culture, whereas *T. augusta*-type trichomonads can be cultured with relative ease (Honigberg, 1963).

The goal of the present study was to survey wild rodents for cecal trichomonads to determine whether the species discovered by Boggild et al. (1996), *T. foetus* or both are present in wild populations of *S. hispidus* and other rodents.

METHODS

Wild rodents were collected with Sherman live-capture traps at several sites in Jackson, Lee, Clarke and Mobile counties in Alabama (Figure 2.1 and Appendix A). Traps were baited with rolled oats and placed in tall-grass fields for a total of 3300 trap nights (a trap night is 1 trap placed at a site for 1 night). Rodents were euthanized in the field and the cecum was removed. Cecal contents were diluted with sterile Hank's Balanced Salt Solution (HBSS) for initial microscopic examination. Samples from each rodent also were placed in 10% formalin for later

microscopic examination and in acetone for later extraction of DNA (Fukatsu, 1999). In addition, cecal samples from domestically-raised rodents were acquired through the Auburn University mammalian physiology laboratory. Two cecal samples from mice caught in Georgia also were acquired. Cecal samples also were taken from 9 captive-raised Mongolian gerbils, and samples of fresh cecal material in HBSS were used for cultures.

Samples of live trichomonads were taken from several specimens and placed in the InPouchTF-Feline culture system (Biomed Diagnostics, White City, Oregon). This system is used for diagnosis of *T. foetus* from feline feces (Gookin et al., 2003). Samples were monitored for growth of trichomonads over several days and were incubated at either 37°C or room temperature. Cultures that survived several days were washed (Appendix B) to remove bacteria and inoculated into a new InPouch.

Samples of cecal material from rodents, either freshly diluted in saline or preserved in 10% formalin, were examined using an Olympus BH-2 Nomarski microscope. Slides containing trichomonads were photographed using a Nikon E8400 camera, and the corresponding samples were chosen for extraction of DNA. Additionally, trichomonads were measured using an ocular micrometer for comparison of size.

RESULTS

Rodents were identified to species in the field before removal of the cecum (Table 2.2). The highest proportion of wild rodents caught was *S. hispidus* (23%) followed by *P. leucopus* (18%). *Peromyscus leucopus* was collected at trapping sites in southern Alabama where *S. hispidus* was absent. *Neotoma floridana* was captured at those sites instead. The greatest variety of rodents was captured in Jackson County, Alabama.

Cecal samples from wild rodents that were placed in the InPouchTF-feline culture system did not reliably provide noticeable cultures of trichomonads. Pouches were overrun quickly by bacteria or fungal spores, and trichomonads were not visible after 1 night of incubation at 37°C.

A second attempt was made to culture cecal trichomonads from captive-raised Mongolian gerbils (*Meriones unguiculatus*). Seven of 9 gerbils contained a large number of cecal trichomonads, which were diluted in sterile HBSS before transfer to the InPouch. Trichomonads reproduced quickly but soon died if left at 37°C for more than 24 hours. Cultures survived longer when placed at 37°C for 1 night and then moved to a dark container at room temperature. The longest surviving culture was from gerbil 8, in which trichomonads survived up to 10 days. This culture was washed using methods listed in Appendix B, placed in a new InPouch and incubated at 37°C overnight.

Flagellates on each slide were categorized as either trichomonads, *Giardia*-like flagellates, or unknown flagellates. Of all cecal samples, 82.7% contained some type of flagellate. More than 90% of the samples from *Peromyscus gossypinus* and *P. leucopus* contained flagellates. More than 75% of the samples from *S. hispidus* and *R. norvegicus* contained flagellates.

Flagellates that resembled trichomonads were measured to determine their length. This was compared to a standard length of 15 µm, the minimum size of the unknown trichomonads reported by Boggild et al (1996). Out of 126 rodent specimens, 59.1% contained large trichomonads (>15 µm), and only 11.0% contained small trichomonads (<15 µm). The species of individuals carrying large trichomonads are listed in Table 2.3.

Large trichomonads were most common in *P. gossypinus* (80%), *M. unguiculatus* (77.8%), *R. norvegicus* (61.9%) and *P. leucopus* (61.9%), all of which are known to carry *T.*

muris. Large trichomonads were in 48.1% of samples from *S. hispidus*, which confirms presence of trichomonads in wild populations.

DISCUSSION

The results confirmed that large trichomonads are present in wild populations of *S. hispidus*, as well as other wild and captive rodents. The morphology of these flagellates suggests that they are in the genus *Tritrichomonas*, and their size suggests that they could be from 2 species, *T. muris* or *T. foetus*. *Tritrichomonas muris* is common among rodents but also is described as being larger than many of the unidentified trichomonads (Levine 1961).

Infections of cats and cattle by *T. foetus* are well known, but this species has not been reported in rodents (Levine, 1961; Gookin et al., 1999). The source of infection by *T. foetus* in felines is unknown, but incidences of it have become increasingly common since 1996 (Gookin et al., 2001). That is also the year that the unknown trichomonad in *S. hispidus* was reported (Boggild et al., 1996). Because cotton rats are common prey for domestic cats (Roberts and Wolfe, 1974), it could be speculated that cats are inoculated by consuming prey. This idea merits further exploration.

Unfortunately, comparisons of size and light microscopy can go only so far toward identification of these trichomonads. In past studies, morphological identification has been used to discern between species of trichomonad (Wenrich and Emerson, 1933; Honigberg, 1963). As methods improved, techniques such as electron microscopy and immunoflourescence microscopy have made morphological studies more detailed (Mattos et al., 1997; Boggild et al., 2002). However, genetic data provide more accurate identification (Kleina et al., 2004). Sequence-level identification has been used to differentiate host-specific isolates of

trichomonads, so this method should be dependable for determining differences in species (Šlapeta et al., 2010). To determine identity of the unknown trichomonad, a genetic study should be done using samples from these rodents.

The present study also has found that trichomonads in rodents can be cultured temporarily with the InPouch TF-feline diagnostic culture system. These culture pouches are designed for specific culture of *T. foetus* and exclude similar species such as *Giardia lamblia* and *P. hominis* (Gookin et al., 2003). Trichomonads from captive gerbils flourished for several days, but samples from wild rodents often were overgrown by bacteria. No test has been conducted out to determine whether *T. muris* would grow in the pouches, probably because this species is notoriously difficult to culture (Honigberg, 1963). Because *T. muris* and feline *T. foetus* are both intestinal flagellates and are the same genus, a culture medium designed for one theoretically could support growth of the other.

There is some limitation in this study in its geographical scope because it reflects only rodents in Alabama (and part of Georgia). Different habitats can support different rodents and their parasites and commensals, so further field studies at other locations should be explored to determine how widespread this parasite may be.

In conclusion, an unknown species of trichomonad has been discovered in wild populations of *S. hispidus* and other rodents. These protists are similar in size to an unknown species of trichomonad discovered in a captive colony of *S. hispidus* in 1996. The size of these flagellates suggests that it is either *T. foetus* or *T. muris*, and some attempts to culture them in a medium designed for *T. foetus* were successful. Further study of these trichomonads at the genetic level is necessary to determine species-level identifications.

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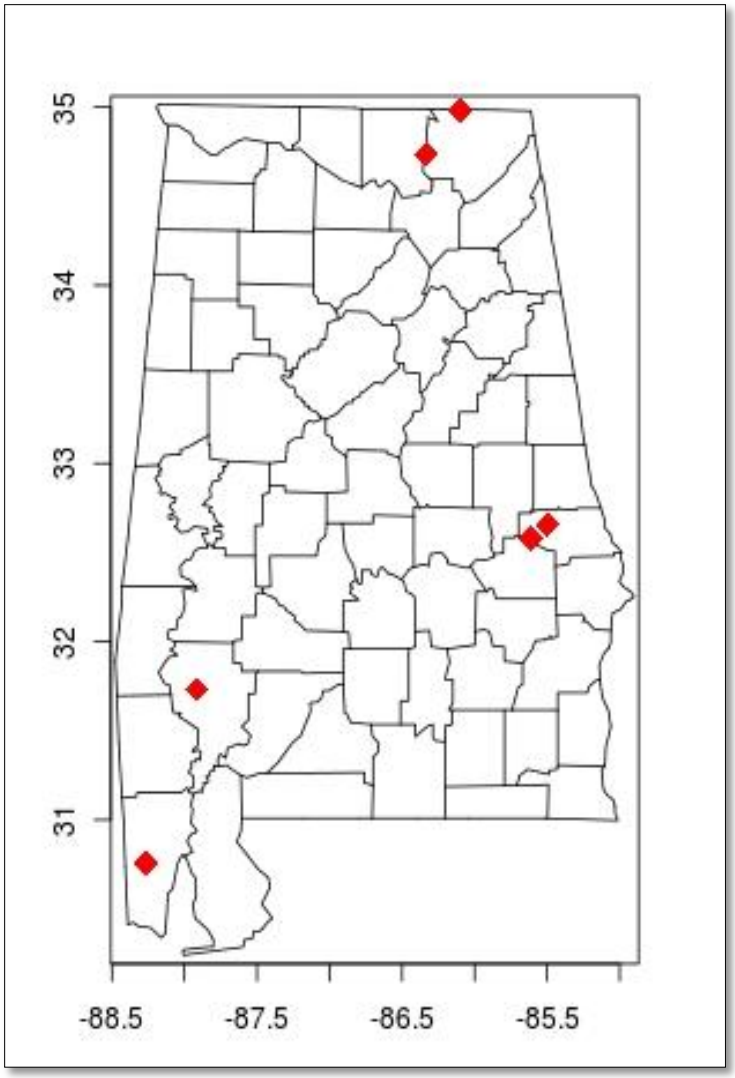

Figure 2.1. Trapping locations in Alabama. (Appendix A.)

Species	Length (µm)	Host
Chilomastix bettencourti	8 – 21	Cecum of mouse, rat, hamster
Giardia muris	7 – 13	Small intestine of mouse, rat, hamster
Hexamita muris	7 – 9	Cecum of mouse, rat, hamster, wild rodents
Monocercomonoides	5 – 15	Cecum of rat, hamster
Octomitus pulcher	6 – 10	Cecum of mouse, rat, hamster, ground squirrel
Pentatrichomonas hominis	9 – 20	Cecum of mouse, rat, hamster, cat, dog, cattle, primates including humans
Retortamonas	4 – 9	Cecum of rat
Spironucleus muris	7 – 9	Cecum of mouse, rat, hamster, wild rodents
Tetratrichomonas microti	4 – 9	Cecum of mouse, rat, vole, other rodents
Trichomitus wenyoni	4 – 16	Cecum of house mouse, brown rat, hamster
Tritrichomonas foetus	10 – 15	Genitalia of cattle; cecum of swine, domestic cat
Tritrichomonas minuta	4 – 9	Cecum of mouse, rat, hamster
Tritrichomonas muris	16 – 26	Cecum of mouse, rat, hamster, gerbil

Table 2.1. Flagellated protists similar in size or shape to those mentioned by Boggild et al. (1996) and their hosts (Baker, 2007; Levine, 1961, 1985).

Species of Rodent	Number	Locations
Meriones unguiculatus	9	Lee County, AL (captive)
Microtus ochrogaster	1	Jackson County, AL
Microtus pinetorum	1	Jackson County, AL
Mus musculus	1	Jackson County, AL
Neotoma floridana	5	Clarke and Mobile counties, AL
Neotoma magister	1	Jackson County, AL
Peromyscus gossypinus	14	Jackson County, AL; Baker County, GA
Peromyscus leucopus	21	Jackson, Clarke, and Mobile counties, AL
Peromyscus maniculatus	1	Jackson County, AL
Peromyscus polionotus	1	Baker County, GA
Rattus norvegicus	42	Lee County, AL (captive)
Sigmodon hispidus	27	Lee and Jackson counties, AL
Tamias striatus	1	Jackson County, AL
Zapus hudsonius	1	Jackson County, AL

Table 2.2. Species of rodents, number collected, and location of capture.

Species of Rodent	Percentage with Flagellates Present	Percentage Infected with Large Trichomonads
Rattus norvegicus (*n* = 42)	76.2	61.9
Sigmodon hispidus (*n* = 27)	81.4	48.1
Peromyscus leucopus (*n* = 21)	90.5	61.9
Peromyscus gossypinus (*n* = 14)	100	85.7
Meriones unguiculatus (*n* = 9)	77.8	77.8
Neotoma floridana (*n* = 5)	100	40
Microtus ochrogaster (*n* = 1)	100	0
Microtus pinetorum (*n* = 1)	100	100
Mus musculus (*n* = 1)	0	0
Neotoma magister (*n* = 1)	100	0
Peromyscus maniculatus (*n* = 1)	100	100
Peromyscus polionotus (*n* = 1)	100	100
Tamias striatus (*n* = 1)	0	0
Zapus hudsonius (*n* = 1)	100	0
Total (*n* = 126)	83.3	59.5

Table 2.3. Species of rodents with number captured, percentage of individuals infected with any type of intestinal flagellate, and percentage infected with large trichomonads (>15 μm long).

CHAPTER III: GENETIC ANALYSIS OF TRITRICHOMONADS OF RODENTS

Trichomonads and other flagellate protists are common in the cecum of rodents (Baker, 2007). These protists include parasites, such as *Pentatrichomonas hominis* and *Giardia lamblia*, that infect other animals. *Tritrichomonas foetus*, a parasite of cattle (*Bos taurus*) and domestic cats (*Felis cattus*), has not been reported in rodents. However, an unidentified protist resembling *T. foetus* was discovered in captive hispid cotton rats (*Sigmodon* hispidus) in 1996, the same year that infections of *T. foetus* in cats became common (Boggild et al., 1996; Gookin et al., 2001). In a previous study, rodents from wild and domestic populations were sampled and their cecal contents were examined microscopically (McNeely, Chapter 2). Trichomonads that resembled those described by Boggild et al. (1996) were in 59.1% of captured rodents, but determination of species of the flagellates was not possible via light microscopy alone. In the present study, I seek to determine the species of these trichomonads by using PCR and DNA-sequence analysis.

Tritrichomonas foetus is a parasite of cattle that causes bovine trichomoniasis, a venereal disease. The disease can cause inflammation of the uterus, spontaneous abortion of the fetus, and sterility of the cow in severe cases (Morgan, 1947). These infections are spread by bulls that carry the parasite in the preputial cavity and in epithelial crypts on the penis (Bondurant, 1985). At the time of infection, trophozoites of *T. foetus* may cause swelling and inflammation of the preputial tissue, but symptoms usually disappear within 2 weeks, after which the bull is usually an asymptomatic carrier (Honigberg, 1978; Felleisen, 1999). There is no treatment for infected bulls and they usually are culled to prevent further infection of the herd (Corbeil et al., 2008). The transmission of *T. foetus* to the cow occurs during breeding, and as the parasite spreads into the uterus and oviducts, an inflammatory response occurs that may prevent fertilization or kill a developing embryo. Abortions usually occur 1 to 16 weeks after breeding and infection

(Morgan, 1947; Bondurant, 1985). The fetus and placenta often will have lesions that can be used to diagnose trichomoniasis after the abortion (Rhyan et al., 1988). If all fetal membranes are not expelled after abortion, the cow may develop chronic endometritis and pyometra, which can cause permanent sterility (Wenrich and Emmerson, 1933). Infected cows usually will mount an immune response that can clear the infection in 2 to 4 months; therefore, cows do not need to be culled. Even so, the cost of losses from *T. foetus* infection in the United States alone is estimated to be hundreds of millions of dollars annually (Speer and White, 1991).

In cats, *T. foetus* causes bowel infections and chronic diarrhea (Levy et al., 2003). The original index case of this infection is unknown, but transmission appears to occur between cats via the fecal-oral route, and the disease occurs most often at densely-populated catteries (Foster et al., 2004). Infected felines can be asymptomatic or have diarrhea with blood, mucus, a foul odor, or a combination of these. Other symptoms include flatulence, tenesmus, and incontinence (Manning, 2010). These symptoms may wane over time, but the cat can still harbor an asymptomatic infection. Most cats are <1 year old when diagnosed with no difference in susceptibility between males and females (Gookin et al., 1999). Symptoms last from 2 days to 3 years, but most cases resolve within 2 years (Foster et al., 2004). Historically, infections of *T. foetus* initially were recognized via light microscopy and PCR testing of feline fecal specimens (Levy et al., 2003), but now infections are detected more commonly using the commercially available InPouch TF culture system that was created originally for detection of *T. foetus* in cattle. Testing of this system has shown that the growth medium cultures *T. foetus* but not other intestinal parasites such as *G. lamblia* and *P. hominis* (Gookin et al., 2003).

Tritrichomonas foetus also has been found in several other species of hosts. *Tritrichomonas suis*, a commensal of the nasal passages and gastrointestinal tracts of swine (*Sus*

scrofa), has been suggested to be the same species as *T. foetus* due to morphological and genetic similarities (Mattos et al., 1997; Tachezy et al., 2002; Lun et al., 2005). Genetic data also have indicated that *Tritrichomonas mobilensis*, a parasite of the intestinal tract of squirrel monkeys (*Saimiri sciureus*), also may be the same species (Kleina et al., 2004). *Tritrichomonas foetus* also has been found occasionally in dogs (*Canis lupus familiaris*) with diarrhea that sometimes have a concurrent infection of *P. hominis*, but its pathogenicity in dogs is unknown (Tolbert et al., 2012).

Because *T. foetus* can be present in such diverse species of hosts, the question arises as to how this flagellate might be transmitted. *Tritrichomonas foetus* has a simple life cycle that consists almost entirely of the trophozoite (active feeding) stage that reproduces by longitudinal fission (Honigberg, 1963). The only notable morphological change *T. foetus* may undergo is the formation of a pseudocyst, which is a spherical, nonflagellated form that offers some protection when an environment becomes inhospitable due to changes such as pH or temperature. In the pseudocyst form, the flagella are withdrawn into vacuoles and the axostyle and costa change to a curved shape (Pereira-Neves et al., 2003). Due to its lack of a true cell wall, the pseudocyst is susceptible to heat and desiccation, which makes it unable to survive for long periods outside of a host. Pseudocysts can survive for a short time in an environment that provides moisture, such as feline urine or wet cat food, but will quickly die in water (Rosypal et al., 2012). The longest recorded survival of *T. foetus* outside of a host is 7 days in a moist fecal sample at room temperature (Hale et al., 2009). Obviously, a pseudocyst cannot persist for lengthy periods outside the host when compared to the longevity of a true cyst or egg. Because the pseudocyst cannot persist outside of the host for long, it may require a vector or reservoir host.

One idea that the present study explores is the potential for rodents to harbor *T. foetus*. For example, one possible reservoir host is the hispid cotton rat, *Sigmodon hispidus*. These rats are native to Neotropical North and South America and range from the Atlantic coast of the United States westward to Arizona and New Mexico and from southern Nebraska to the northern coast of South America (Cameron and Spencer, 1981). These rodents occupy open field habitats with tall grasses that supply them with cover and food (Fleharty and Olson, 1969; Cameron and Spencer, 1981). Thus, cotton rats are likely to be in cattle-grazing habitats. They are active both day and night (Cameron and Spencer, 1981) and due to their large populations are likely to be a common prey for cats. This implies that if *T. foetus* is carried by the cotton rat or any other prey rodent, it could have direct access to the digestive tract of feline hosts. Additionally, trichomonads similar in size to *T. foetus* were identified in *S. hispidus* in 1996 (Boggild et al., 1996) and in another study (Chapter 2) where none had been seen before (Doran, 1954).

Due to economic costs of bovine trichomoniasis, much research is concerned with diagnostic methods to identify the parasite. Genetic analysis is more objective than morphological examination, but it requires sequences that are highly conserved but variable enough to show a difference between species. The gene for 5.8S ribosomal RNA is a good identification site among trichomonads because it is highly conserved, but it also is surrounded by internal transcribed spacer (ITS) regions 1 and 2, which can vary without any selective effect. Primers that targeted this site in *T. foetus* were designed by Felleisen et al. (1998), and additional nested primers for this region were designed by Gookin et al. (2002; Table 3.1). These primers are still in use by veterinary labs for the diagnosis of *T. foetus* in bovine and feline patients.

Some evidence indicates that there are genetic differences between bovine and feline isolates of *T. foetus.* Slapeta et al. (2010) discovered a single conserved polymorphism in the

ITS2 region that differs between isolates. Additional differences have been found on the gene for elongation factor 1-alpha (EF-1α), a common protein that previously had been identified in *Trichomonas vaginalis*, and on the gene for cysteine protease 8, a protein involved in host-parasite interactions (Reinmann et al., 2012; Sun et al., 2012). If *T. foetus* occurs in rodents, these genetic differences could indicate whether it is more closely related to the bovine or feline isolates.

METHODS

Wild rodents were collected with Sherman live-capture traps at several sites in Jackson, Lee, Clarke and Mobile counties in Alabama (see Chapter 2). Rodents were euthanized in the field and the cecum was removed. Cecal contents were diluted with sterile Hank's Balanced Salt Solution (HBSS) for initial microscopic examination. Samples from each rodent were placed in 10% formalin for later microscopic examination and in acetone for later extraction of DNA (Fukatsu, 1999). In addition, cecal samples from domestically-raised rodents were acquired through the Auburn University mammalian physiology laboratory. Two cecal samples from mice caught in Georgia were acquired as well. Cecal samples also were taken from 9 captive-raised Mongolian gerbils.

DNA was extracted from acetone-preserved specimens using the Qiagen DNeasy kit (Appendix C). Extracted DNA was analyzed with a Nanodrop 1000 spectrophotometer (Thermo Scientific) to determine DNA content before PCR testing. All PCRs were performed using Dreamtaq™ PCR mastermix (Fermentas, Glen Burnie, Maryland), a pre-optimized solution of *Taq* polymerase, buffers, and dNTPs. Single primer PCR was done in 50-µl aliquots using 25 µl mastermix, 21 µl DNase-free H2O, 1 µl of each primer (10µM), and 2 µl of template DNA.

Each set of PCR reactions was accompanied by a negative control that contained sterile water. Each primer set was tested with positive controls from cultures of *T. foetus* from feline and bovine hosts (designated AUTF-12 and TF-D1, respectively) (Skirrow and Bondurant, 1990; Stockdale et al., 2006), as well as *T. mobilensis* (ATCC 50116). A TC-312 thermal cycler (Techne) was used with the temperature protocols below.

Primers TFR1 and TFR2 (Felleisen, 1997) were used to confirm presence of trichomonads using the following protocol: initial denaturation at 95°C for 10 minutes; 40 cycles of denaturation for 30 seconds at 95°C, annealing for 30 seconds at 66°C, extension for 90 seconds at 72°C; final extension for 15 minutes at 72°C. Specimens that tested positive with these primers were then tested with primers TFR3 and TFR4 to confirm presence of *T. foetus*. The thermal cycling protocol for these species-specific primers was used directly from Felleisen et al. (1998).

Additionally, protocols for nested PCR using TFR3 and TFR4 and the TFITS primers published by Gookin et al. (2002) were used to verify *T. foetus* in positive specimens. We prepared 50-µl aliquots that contained 25 µl Dreamtaq mastermix, 10 µl DNase-free H_20, 0.05µM each of TFR3 and TFR4, 6.25µM each of TFITS-F and TFITS-R, and 2 µl of template DNA. For initial amplification using the TFR3/TFR4 primers, samples were denatured at 95°C for 5 minutes and then run through 50 amplification cycles of 30 seconds at 95°C, 30 seconds at 67°C, and 45 seconds at 72°C, followed by a final extension at 72°C for 5 minutes. Then, the same samples were run again with the same 95°C initial denaturation and 50 cycles using a lower 57°C annealing temperature and extensions of only 30 seconds. These primers produced a 208-bp product that indicated the presence of *T. foetus.*

All PCR products were electrophoresed in 2% agarose gels containing ethidium bromide and TAE buffer fluid (Appendix D). Each set of primers produced a different-sized product as listed in Table 3.1.

After results of PCR were confirmed, specimens that tested positive for *T. foetus* with primers TFR3 and TFR4 were sequenced using the same primers. Initial sequencing was done at the Auburn University Genomics and Sequencing Laboratory. Before sequencing, PCR templates were diluted with 300 µl Nanopure H20 and filtered using Amplicon Ultra centrifugal filters (Millipore) to remove excess primers. Final sequencing was done through Lucigen Corp. (Middleton, Wisconsin), and the products of PCR were cleaned as part of the sequencing service.

Upon receiving sequence data, base calls for each chromatogram were checked using BioEdit software (T. Hall, http://www.mbio.ncsu.edu/bioedit/bioedit.html). Forward and reverse sequences were aligned to provide a more accurate composite sequence. These sequences were aligned with sequences from feline and bovine isolates of *T. foetus* for comparison. BLAST sequence alignment also was used to identify the species of each sequence with >90% confidence. Finally, a phylogenetic tree was created to determine the degree to which each specimen was related to other species of trichomonad. A maximum-parsimony tree with bootstrap support (1000 repetitions) was created using the maximum-parsimony function in MEGA (Tamura et al., 2011).

To ensure further support of the phylogeny, further PCR and sequencing was done using the gene for elongation factor 1-alpha (Table 3.1). Primers EF-1α feline F and EF-1α R were used to amplify the gene in trichomonad DNA from cecal specimens of rodents. Of the 2 forward primers designed by Reinmann et al. (2012), the feline forward primer was chosen because this forward primer amplifies *T. suis*, *T. mobilensis*, and both genotypes of *T. foetus*,

whereas the bovine forward primer amplifies only the bovine genotype of *T. foetus*. The PCR protocol described by Reinmann et al. (2012) did not amplify DNA of trichomonads from rodents, possibly due to my use of a different mastermix. A new protocol was determined as follows: 15 µl Dreamtaq mastermix, 2 ul each primer at 10 µM concentration, 17 µl H_2O and 4 µl template DNA per reaction, plus 1 µl Q-solution (Qiagen, Germantown, Maryland) per 5 reactions. Initial denaturation occurred at 95°C for 15 minutes and was followed by 40 cycles of denaturation at 94°C for 15 seconds, annealing at 54°C for 15 seconds, and extension at 72°C for 60 seconds. Final extension at 72°C lasted 5 minutes.

PCR products were resolved in 2% agarose gels as described above. Due to amplification of different sizes of DNA, bands were cut from the gel and DNA was extracted using the Zymoclean™ Gel DNA Recovery Kit (Zymo Research Corporation, Irvine, California). Positive specimens from phylogenetic groups of *T. foetus* and *T. muris* were chosen for sequencing. The EF-1α sequences were aligned and excess length was removed from 2 of the sequences from rodents and 3 sequences of the outliers. The EF-1α sequence of each was concatenated with the corresponding 5.8S sequence. These combined sequences were used to create a maximum parsimony tree with bootstrap support (1000 repetitions).

RESULTS

PCR for general trichomonads was positive in 61.1% of rodents (n = 77 of 126). Hosts that tested positive for general trichomonads included *Rattus norvegicus* (n = 24 of 42), *S. hispidus* (n = 16 of 27), *Peromyscus gossypinus* (n = 13 of 15), *Peromyscus leucopus* (n = 11 of 21), *Meriones unguiculatus* (n = 7 of 9), *Neotoma floridana* (n = 3 of 5), *Microtus pinetorum* (n = 1 of 1), *Peromyscus maniculatus* (n = 1 of 1), and *Peromyscus polionotus* (n = 1 of 1). No

trichomonad was detected in *Microtus ochrogaster*, *Mus musculus*, *Neotoma magister*, *Tamias striatus*, or *Zapus hudsonius*, although only 1 cecal sample was available from each of these species.

Rodent specimens were tested further for presence of *T. foetus*, which was found in 28.6% of specimens using primers TFR3 and TFR4 (*n* = 36 of 126; Figure 3.1). TFITS primers actually amplified DNA in fewer specimens (*n* = 31) than the *T. foetus*-specific TFR primers 3 and 4. A comparison of PCR results is shown in Figure 3.2.

All 36 sequences that tested positive for *T. foetus* with TFR3 and TFR4 were sequenced with those primers. Unfortunately, due to mixed populations of flagellates in the cecum of rodents, many of the sequences produced a cluttered chromatogram. In many cases, the sequence was unreadable up to the 100th base call. Consensus sequences were formed for the majority of specimens, but a clear consensus was not possible in 2 cases.

According to BLAST, 14.3% (*n* = 18 of 126) of all cecal specimens were identified as *T. foetus* with >90% maximum identity. The majority of these were from *R. norvegicus* (35.3%; *n* = 6 of 18) and *S. hispidus* (35.3%). Sequences matching *T. foetus* also were in samples from *P. leucopus* (*n* = 3 of 21) and *P. gossypinus* (*n* = 3 of 15). An additional 16 specimens were identified as *T. muris* with >83% maximum identity. Hosts for *T. muris* included *M. unguiculatus* (*n* = 7 of 9), *S. hispidus* (*n* = 4 of 27), *P. leucopus* (*n* = 2 of 21), *P. gossypinus* (*n* = 1 of 15), *P. maniculatus* (*n* = 1 of 1), and *M. pinetorum* (*n* = 1 of 1). Surprisingly, none of the captive-raised rats (*R. norvegicus*) tested positive for *T. muris*. A comparison of BLAST results for each species of host is in Table 3.2.

Sequences were aligned and MEGA was used to generate a maximum-parsimony tree (Figure 3.3). This tree provided a clear separation of sequences associated with *T. foetus* and

those associated with *T. muris*. There did not seem to be any specific association with either isolate of *T. foetus*.

To determine relatedness of trichomonads from more than one locus, the EF-1α gene was amplified from several samples and then sequenced. PCR amplicons were generated in 2 of the samples that grouped with *T. foetus* in the previous phylogeny, 3 of the samples that grouped with *T. muris*, and 3 positive control samples (*T. foetus*-feline isolate, *T. foetus*-bovine isolate, and *T. mobilensis*). The 2 samples from *M. unguiculatus* amplified products about 100 bp larger than control samples (Figure 3.4). An even larger amplicon was formed in AL021 (*S. hispidus*); however, this amplicon did not produce a quality sequence.

Sequences for the EF-1α gene were concatenated with those from the ribosomal RNA genes before the production of a second tree (Figure 3.5). Chromatograms for these sequences were clearer than those from the ITS1-5.8S-ITS2 region. In phylogeny, 1 of the samples that previously had been associated with *T. foetus* (AL013) was separate from both the *T. foetus* and *T. muris* groups. There was also a greater amount of genetic difference between trichomonads from gerbils (AL0G1 and AL0G2) and the other sample *T. muris* (AL021) than was indicated by the previous phylogeny (Figure 3.3).

DISCUSSION

Results of this study confirm the presence of *T. foetus* in 14.3% of surveyed rodents. The parasite was in both wild (9.5%; n = 12 of 126) and captive-raised rodents (4.7%; n = 6 of 126). These data indicate a possible reservoir population from which cats, cattle, or both could be infected. Presence of this reservoir would explain the mode of transmission through which *T. foetus* could be transferred from cattle to cats, which previously has eluded investigators.

Phylogenetic data do not indicate whether these trichomonads are more closely related to feline isolates of *T. foetus* or to bovine isolates. This raises several questions about transmission of *T. foetus*. If the feline isolate of *T. foetus* was obtained originally from a rodent and later adapted to felines, it would require significant time and selective pressure to change the DNA of conserved regions such as ITS2. That also would mean that feline trichomoniasis has spread only from cat to cat, without using a rodent host. Conversely, it also is possible that feline trichomoniasis is rare in Alabama. A study published by Stockdale et al. in 2009 found *T. foetus* in only 4 of 69 cats from Alabama as opposed to 5 of 10 cats surveyed in California. More data from other states would be required to determine prevalence of *T. foetus* in rodents, as well as prevalence of its different genotypes.

It is also worth considering that *T. foetus* could have spread originally from rodents or other hosts to cattle, as well as to cats. Figure 3.5 depicts close association between 2 bovine isolates and 2 feline isolates, but neither pair is more closely related to *T. foetus* taken from the cecum of *S. hispidus*. If *T. foetus* is uncommon in rodents and does not match either of the parasitic genotypes, it may be acquired from a different species. Swine are known to carry commensal *T. foetus* in the cecum (Tachezy et al., 2002); rodents easily could consume porcine feces that contain the parasite. Swine also gain parasites by eating rodents (Weinman and Chandler, 1954), so it also would be possible for rodents to contribute to further infections of *T. foetus* in swine. The flagellate appears to be commensal in both species. If *T. foetus* from swine and rodents are closely related, the parasitic nature of bovine and feline isolates could be a derived feature. Further work on the DNA of *T. foetus* from different hosts may be required to determine how closely each strain is related, and which hosts might provide a source of the

parasite for others. Cross-infection studies would be especially useful in determining whether the *T. foetus* in rodents can parasitize bovine or feline hosts.

There was an interesting anomaly in our study of the EF-1α locus. One of the trichomonads that previously had matched with the conserved ITS1-5.8S-ITS2 region of *T. foetus* did not match it at the EF-1α locus. The EF-1α sequence did not match any parabasalid species on BLAST, and in phylogeny, it appears to be a distant relative of the putative samples of *T. muris*. The chromatogram for this sequence was clean and did not indicate amplification of multiple sequences. This may indicate that there is a separate genotype of *T. foetus* in some rodents, or that the EF-1α gene from a different trichomonad was amplified more than that of *T. foetus*.

This study also found that another trichomonad, which appears to be *T. muris*, was amplified in PCR and sequencing by the primer sets typically used in veterinary diagnosis of *T. foetus*. *Tritrichomonas muris* is notoriously difficult to culture; thus, few dependable genetic sequences are available in GenBank. All available sequences of the ITS1-5.8S-ITS2 region are taken from trichomonads removed directly from the cecum of wild yellow-necked mice (*Apodemus flavicollis*) in the Czech Republic (Cepicka et al., 2006). Whether these trichomonads were examined morphologically before being classified as *T. muris* is unknown. The only other record of *T. muris* in GenBank is a 28S rDNA gene provided by Viscogliosi et al. (1993), which was sequenced after confirmation of its species by light and electron microscopy.

It also is noteworthy that trichomonads from captive-raised gerbils all aligned with *T. muris* phylogenetically, yet they were cultured easily in the InPouch TF-feline culture system for several days (McNeely, Chapter 2). It is possible that these flagellates are a different species of

trichomonad. The difference in EF-1α sequences between trichomonads from gerbils and those from other rodents also supports this idea.

Even among the putative samples of *T. muris* from other rodents, none of them was collected from captive-raised *R. norvegicus*. This seems odd in comparison to reports that *T. muris* is so common in laboratory animals that it rarely is reported in surveys for parasites (Baker, 2007). It also is noteworthy that many rodents contained large trichomonads that were not amplified by the *T. foetus*-specific PCR. Further examination of these flagellates may be required to determine which trichomonads are actually *T. muris*.

In conclusion, *T. foetus* has been discovered in the cecum of both wild and domestic rodents. This indicates that rodents are a reservoir for trichomonads from which feline trichomoniasis, bovine trichomoniasis, or both could have arisen.

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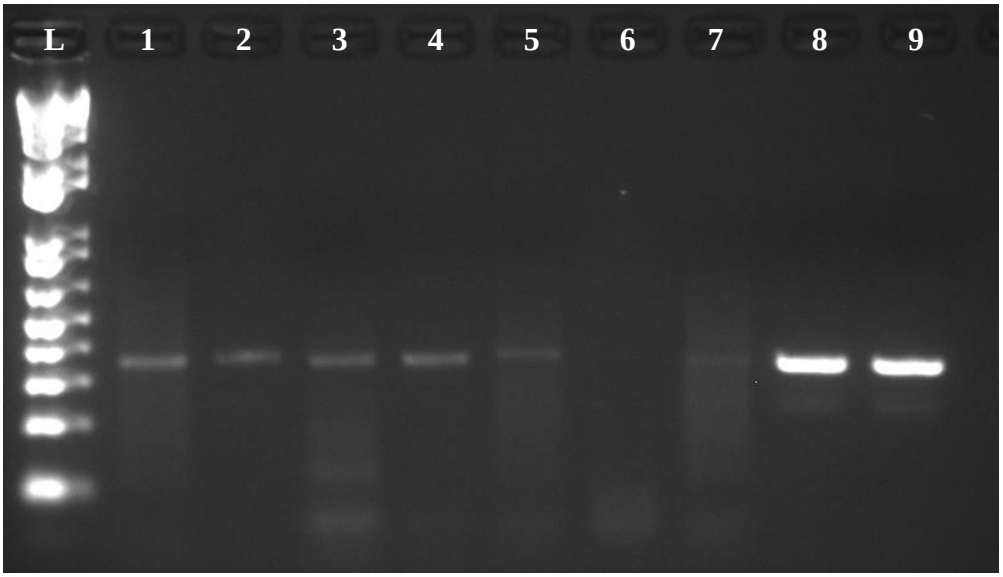

Figure 3.1. Photograph of PCR results from amplification using primers TFR3/TFR4. Lane L, 1kbp ladder; lane 1, cecal sample from *Sigmodon hispidus*; lane 2, from *Rattus norvegicus*; lane 3, from *Peromyscus leucopus*; lane 4, from *Peromyscus gossypinus*; lane 5, from *Microtus pinetorum*; lane 6, sterile water; lane 7, cultured isolate of *Trichomonas vaginalis*; lane 8, cultured feline isolate of *Tritrichomonas foetus*; lane 9, cultured bovine isolate of *T. foetus*.

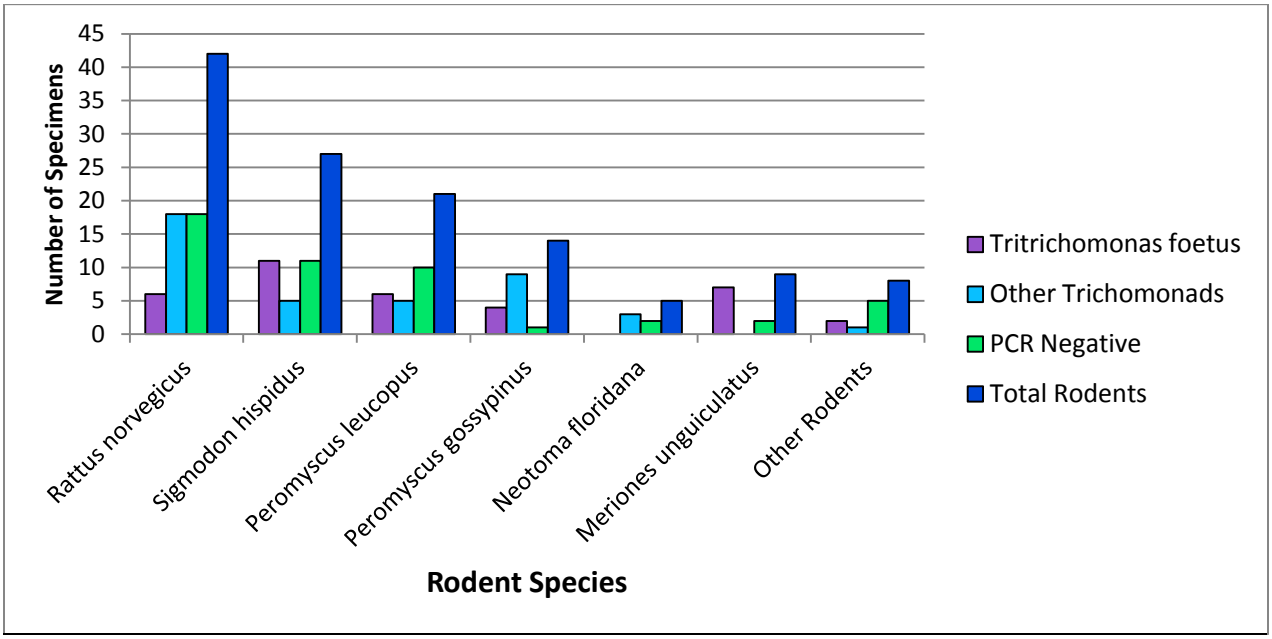

Figure 3.2. Results of PCR for cecal samples from each rodent host. *Tritrichomonas foetus* determined by primers TFR3 and TFR4; Other trichomonads determined by primers TFR1 and TFR2.

Figure 3.3. Maximum-parsimony tree for ITS1-5.8S-ITS2 sequences of cecal samples, as well as cultured control isolates(*) and GenBank sequences(**).

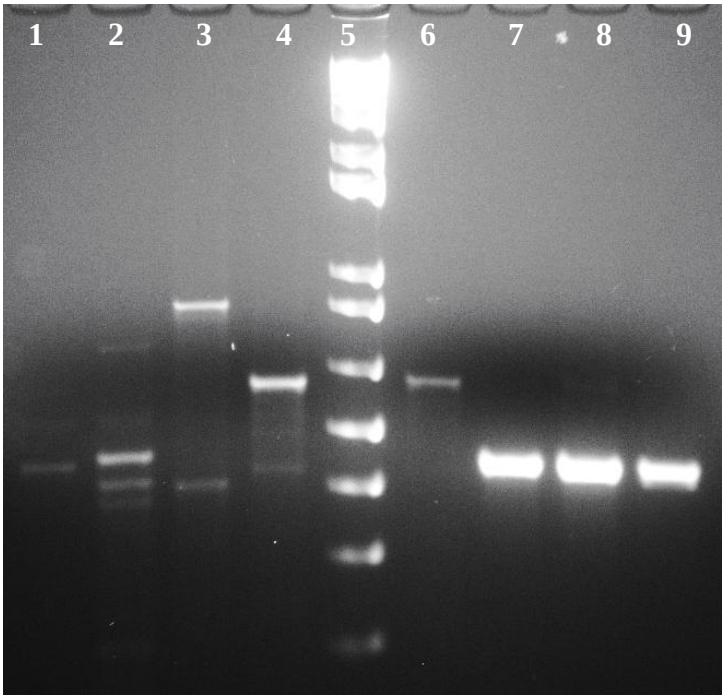

Figure 3.4. Gel containing positive EF-1α samples. Lane 1, cecal sample from AL011 (*Sigmodon hispidus*); lane 2, from AL013 (*S. hispidus*); lane 3, from AL021 (*S. hispidus*); lane 4, from AL0G1 (*Meriones unguiculatus*); lane 5, 1kbp ladder; lane 6, from AL0G4 (*M. unguiculatus*); lane 7, cultured feline isolate of *Tritrichomonas foetus*; lane 8, cultured bovine isolate of *T. foetus*; lane 9, cultured isolate of *Tritrichomonas mobilensis*.

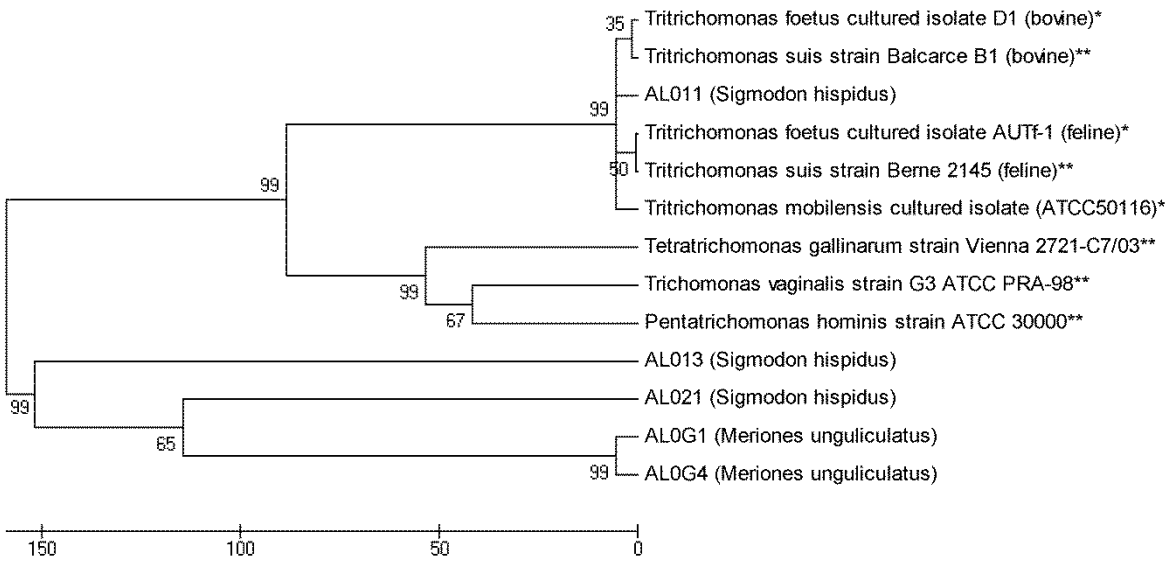

Figure 3.5. Concatenated phylogeny of select trichomonad samples, combining sequences from the ITS1-5.8S-ITS2 region and the EF-1α region. Sequences AL011 and AL013 were associated with *Tritrichomonas foetus* in a previous phylogeny and sequences AL021, AL0G1, and AL0G4 were associated with *Tritrichomonas muris* (Figure 3). Additional sequences from cultured isolates (*) and GenBank (**) included.

Primer	Sequence (5' to 3')	Product Size (bp)	Reference
TFR1	TGCTTCAGTTCAGCGGGTCTTCC	372	Felleisen, 1997
TFR2	CGGTAGGTGAACCTGCCGTTGG		
TFR3	CGGGTCTTCCTATATGAGACAGAACC	347	Felleisen et al., 1998
TFR4	CCTGCCGTTGGATCAGTTTCGTTAA		
TFITS-R	GCAATGTGCATTCAAAGATCG	208	Gookin et al., 2002
TFITS-F	CTGCCGTTGGATCAGTTTCG		
EF-1α feline F	CAACCTCGGATGGTACAC	460	Reinmann et al., 2012
EF-1α R	GCGATGTGAGCTGTGTGGC		

Table 3.1. Primers used for identification of trichomonads and size of product DNA amplified by each pair. TFR 1 and 2 were used to identify the presence of trichomonads; TFR 3 and 4 and the TFITS primers were used to identify *Tritrichomonas foetus*; EF-1α primers were used to sequence the EF-1α locus for phylogenetic purposes.

Species of Host	Percentage positive for *Tritrichomonas foetus*	Percentage positive for *Tritrichomonas muris*
Sigmodon hispidus (*n* = 27)	22.2	14.8
Rattus norvegicus (*n* = 42)	14.3	0
Peromyscus leucopus (*n* = 21)	14.3	9.5
Peromyscus gossypinus (*n* = 14)	21.4	7.1
Peromyscus maniculatus (*n* = 1)	0	100
Meriones unguiculatus (*n* = 9)	0	77.8
Microtus pinetorum (*n* = 1)	0	100
Total (*n* = 126)	14.3	12.7

Table 3.2. Host of each sequenced specimen and species of tritrichomonad determined by BLAST.

Appendix A: Trapping Locations and Dates

Lee County

10-11 September 2010: Auburn University Fisheries Unit, Farmville Road, 4 miles N of Auburn, AL

N: 32° 39.816’ W: 85° 30.201’

3-11 May 2011: Auburn University Aviaries, Bee Lab Rd off Lem Morrison Drive, Auburn, AL

N: 32° 35.934’ W: 85° 30.052’

9-10 September 2011: Auburn University Fisheries Unit, Farmville Road, 4 miles N of Auburn, AL

N: 32° 39.816’ W: 85° 30.201’

18 September 2011: Lee County Rd 188, off Macon County Rd 54

N: 32° 34.676’ W: 85° 36.588’

Jackson County

2-3 October 2010: The Nature Conservancy Office, Paint Rock River, near Gurly, AL

N: 34° 44.328’ W: 86° 19.560’

8-10 October 2010: Walls of Jericho, Skyline Wildlife Management Area, near Scottsboro, AL

N: 34° 58.978’ W: 86° 5.759’

16-17 October 2010: Walls of Jericho, Skyline Wildlife Management Area, near Scottsboro, AL

N: 34° 59.024’ W: 86° 5.679’

22-24 October 2010: Walls of Jericho, Skyline Wildlife Management Area, near Scottsboro, AL

N: 34° 59.031’ W: 86° 5.687’

30 September-2 October 2011: The Nature Conservancy Office, Paint Rock River, near Gurly,

AL

N: 34° 44.469’ W: 86° 19.514’

15-17 October 2011: Walls of Jericho, Skyline Wildlife Management Area, near Scottsboro, AL

N: 34° 59.018’ W: 86° 5.743’

Clarke County

16-20 March 2011: Skipper Farm, Mile 36, Highway 84W, Zimco, near Grove Hill, AL

N: 31° 42.974’ W: 87° 53.352’

Mobile County

16-21 May 2011: Green Acres Nursery, 9730 Roberts Lane W, Semmes, AL

N: 30° 28.551’ W: 88° 15.843’

Appendix B: Culture Rinsing Methods

1. Transfer total contents of pouch to 2 microcentrifuge tubes.
2. Centrifuge at less than 500 RCF for 2 minutes.
3. Decant, resuspend sediment in 1-3 mL of warm PBS.
4. Repeat centrifugation.
5. Decant, remove sediment to new InPouch.

APPENDIX C: Instructions Accompanying the Qiagen DNeasy Kit

Protocol: Purification of Total DNA from Animal Tissues (Spin-Column Protocol)

This protocol is designed for purification of total DNA from animal tissues.

Procedure

1. Dilute cecal material with HBSS and place 200 µl of the resulting fluid in a 1.5-ml microcentrifuge tube. Add 180 µl Buffer ATL.

2. Add 20 µl proteinase K. Mix thoroughly by vortexing, and incubate at 56°C until the cells are completely lysed (1 hour). Vortex occasionally during incubation to disperse the sample.

3. Vortex for 15 seconds. Add 200 µl Buffer AL to the sample, and mix thoroughly by vortexing. Then add 200 µl of ethanol (96–100%), and mix again thoroughly by vortexing.

4. Pipette the mixture from step 3 (including any precipitate) into the DNeasy Mini spin column placed in a 2 ml collection tube (provided). Centrifuge at 6000 x g (8000 rpm) for 1 min. Discard flow-through and collection tube.

5. Place the DNeasy Mini spin column in a new 2 ml collection tube (provided), add 500 µl Buffer AW1, and centrifuge for 1 min at 6000 x g (8000 rpm). Discard flow-through and collection tube.

6. Place the DNeasy Mini spin column in a new 2 ml collection tube (provided), add 500 µl Buffer AW2, and centrifuge for 3 minutes at 20,000 x g (14,000 rpm) to dry the DNeasy membrane. Discard flow-through and collection tube.

7. Place the DNeasy Mini spin column in a clean 1.5-ml microcentrifuge tube, and pipette 200 µl Buffer AE directly onto the DNeasy membrane. Incubate at room temperature for 1 min, and then centrifuge for 1 min at 6000 x g (8000 rpm) to elute.

APPENDIX D: TAE and Agarose Gel

10x TAE buffer:

48.4g Tris Base

7.4g Disodium EDTA

16.4g Sodium Acetate Anhydrous

17mL Glacial Acetic Acid

dH_2O to 1 Liter

Dilute to 1x TAE using Nanopure H_2O

2% Agarose Gel:

100 ml TAE (1x)

2.0 g Agarose (Genetic Analysis grade, BP1356-100, Fisher Scientific)

8 µl Ethidium Bromide (10 mg/mL, IBI Scientific)