MOLECULAR TYPING OF *GIARDIA LAMBLIA* IN HUMANS AND DOGS AND EVIDENCE FOR SEXUAL RECOMBINATION

By

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A Dissertation Submitted to the Faculty of the

DEPARTMENT OF VETERINARY SCIENCE AND MICROBIOLOGY

In Partial Fulfillment of the Requirements
For the Degree of

DOCTOR OF PHILOSOPHY

MAJOR IN PATHOBIOLOGY

In the Graduate College

THE UNIVERSITY OF ARIZONA

2006

THE UNIVERSITY OF ARIZONA
GRADUATE COLLEGE

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ACKNOWLEDGMENTS

I thank my husband and soul mate Kerry for his encouragement and support in the fulfillment of this degree. He has been an invaluable partner as a scientist and as a partner in life. I look forward to many years of pondering the questions of science with him by my side.

I thank Dr. Chuck Sterling for choosing me to be a part of his research team and his patience and insight as my mentor. I cannot thank him enough for giving me the opportunity to prove myself as a contributor to academia.

I thank my doctoral committee member Dr. Rod Adam for his guidance in the detailed aspects of *Giardia* genetics, which went beyond the requirements of a doctoral committee member.

I thank my doctoral committee members, Drs. Helen Jost, Lynn Joens, and Donna Wolk for their contributions to my research development as a scientist-in-training. I thank Dr. Robert Gilman, Dr. Vitaliano Cama, Lilia Cabrera, and Carmen Guardamina for their assistance with the Peruvian study site. I also thank Dr. Michael Worobey for his assistance with algorithmic analysis and the presentation of said data.

I would also like to acknowledge the financial support of BRAVO! and ARCS.

DEDICATION

To

Kerry Kevin Cooper

My Husband

And

Soul Mate

And

My Family,

Dan and Ida,

Daniel,

Ray and Shirley

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ABSTRACT

Giardia lamblia is a eukaryotic parasite that causes diarrhea in humans worldwide. Diarrheal diseases cause stunting and mental retardation in children in developing nations, therefore it is important to understand the molecular epidemiology of *G. lamblia.* Compounding this, it is not clear if companion animals such as dogs contribute to infections in humans through zoonotic transmission. The genotypes of *G. lamblia* that have been found in humans are A1, A2 and B, while those in dogs have been on rare occasions all three human genotypes, but largely C and D, which have only been reported in dogs and appear to be species-specific. The molecular epidemiology of *G. lamblia* in humans and dogs was assessed in an endemic region of Lima, Peru. With one exception, dogs were found to harbor the C and D dog genotypes of *G. lamblia*. A single family dog was found to harbor a human genotype of *G. lamblia.* A2 and B genotypes of *G. lamblia,* but not A1, were found in humans in the endemic region. Previous literature reported that A2 and B typing within genotype tools were available, however the A2 samples from the endemic region could not be distinguished from one another through nucleotide polymorphism sequence analysis. A molecular typing technique was developed to type A2 samples. The extensive sequence analysis performed on two chromosomes of *G. lamblia*, yielded different phylogenetic tree groupings for the same samples. This lead to algorithmic analysis, which demonstrated a significantly high probability that meiotic recombination is occurring in the A2 samples of *G. lamblia.* As *G. lamblia* is largely believed to be asexual, the conclusion of doctoral research

performed in this study yielded controversial, yet significant evidence that sex in *G. lamblia* A2 genotype samples is indeed occurring.

1.0 INTRODUCTION

1.1 Preface

Giardia lamblia is a fascinating protozoan parasite, and the author's fondness began with a slide of a trophozoite under light microscopy. The risks of anthropomorphizing a microscopic organism notwithstanding, the two nuclei peer at one from the slide, and how can one resist *G. lamblia*'s comical smile and enticing dare to better understand it?

1.2 History

Giardia lamblia has the distinction of being described by the father of microbiology, Antony van Leeuwenhoek in 1681. Almost 200 years would pass before the Czech physician Vilem Lambl would describe it again in 1859. In 1882 Kunstler proposed the genus *Giardia* for a flagellated microorganism found in tadpoles, which was accepted and is in use to the present day(Boreham *et al.*, 1990). Francis Filice published his *Giardia* taxonomic designations based upon morphology which will be discussed in more detail in a later section (Filice, 1952).

There were two important publications that led to subsequent understanding of the parasite. Rendtorff studied transmission of *G. lamblia* cysts in "volunteer" prisoners and determined that as little as ten cysts can cause infection in humans (Rendtorff, 1954). Karapetyan was the pioneer of *in vitro* culturing of *G. lamblia* (Karapetyan, 1962). Meyer successfully cultivated *G. lamblia* axenically, or in pure culture, for samples

obtained from a rabbit, chinchilla, and cat (Meyer, 1970). Axenic cultivation has allowed for better understanding of the biology of this parasite.

Interestingly, *G. lamblia* cysts have been detected in ancient human coprolites in Peru. One sample dated between 2,375-1,525 B.C.E. and a second sample dated between 500-900 C.E. (Ortega & Bonavia, 2003).

1.3 Taxonomy

Giardia lamblia (syn. *G. intestinalis*, *G. duodenalis*) is a unicellular eukaryote that has four pairs of flagella (Adam, 2001). It is a member of binucleate microorganisms known as diplomonads, which are considered protozoa (Adam, 2000).

Filice detected morphological differences in *Giardia* spp. and designated *G. duodenalis* in humans, *G. agilis* in amphibians, and *G. muris* in mice. The species name of G. lamblia is currently under debate, as it is designated *lamblia, intestinalis,* or *duodenalis* depending upon the author. In this work the microorganism will be identified as *Giardia lamblia.*

Within the *G. lamblia* morphologic grouping, three additional species were subsequently identified by electron micrographic criteria; *G. psittaci* in budgerigars (Erlandsen & Bemrick, 1987), *G. ardeae* in birds (Erlandsen *et al.*, 1990), and *G. microti* in muskrats and voles (van Keulen *et al.*, 1998).

Even within the *Giardia* isolates that are still designated as *G. lamblia* by morphologic criteria, there are substantial differences that can be demonstrated by criteria based on genotyping, host specificity, and biological differences. Molecular criteria have

been used to divide these isolates into genotypes A through G. The human genotypes of *G. lamblia* are A1, A2 and B. There are two dog genotypes, C and D. Genotype F is found in cats, genotype E is found in hoofed livestock, and G is found in rats.

Table 1. Genotypes of *Giardia lamblia*

Genotype	**Host Range**
A1	Humans, livestock, cats, dogs, beavers, guinea pigs, other primates
A2	Humans, dogs
B	Humans, other primates, dogs, beavers, other wildlife
C/D	Dogs
E	Cattle, sheep, pigs, goats, alpaca
F	Cat
G	Rats

1.4 Life Cycle

The life cycle of *G. lamblia* consists of two stages: cysts and trophozoites (Figure 1). The cysts are environmentally hardy (Adam, 1991) and are ingested by an unsuspecting host. The acidic environment of the host stomach followed by the neutral pH of the small intestine triggers excystation of the cyst. This causes the trophozoites to emerge and begin feeding and multiplying in the host small intestine. Trophozoites must encyst to be excreted with the feces and infect a new host through fecal-oral transmission (Farthing, 1997).

Trophozoites range from 12 to 15 μm in length and 5 to 9 μm in width. The cytoskeleton of trophozoites consists of a median body, four pairs of flagella, and a ventral disk. There are two nuclei. Trophozoites reside in the small intestine, attaching

to the intestinal wall mechanically via the ventral adhesive disk. The flagella appear to be critical for motility, but do not seem to play a role in attachment (Adam, 2001).

Encystation is estimated to take 10 to 16 hours, during which cyst wall proteins are expressed and two trophozoites with four nuclei form that have not yet undergone cytokinesis (Adam, 2001; Erlandsen *et al.*, 1996). During encystation the trophozoites become rounder, detach, and lose mobility, becoming refractile (Gillin *et al.*, 1996). Encystation-specific secretory vesicles can be observed by light microscopy, which serve to convey regulated antigens to the cyst wall (Faubert *et al.*, 1991; Reiner *et al.*, 1989).

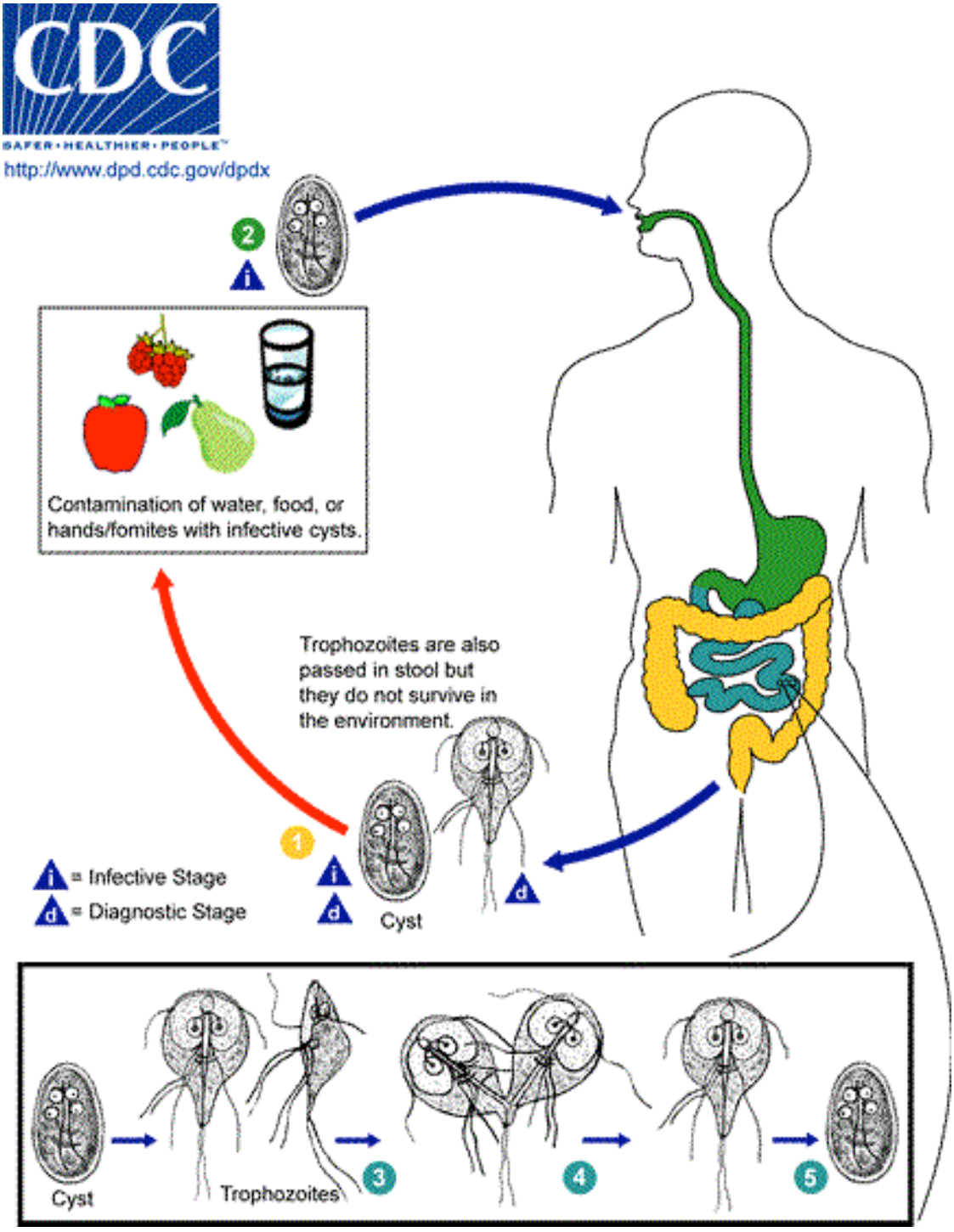

Figure 1. Life cycle of *Giardia lamblia* (from www.cdc.gov/)

1.5 Genomics and Physiology

The genome of *G. lamblia* is approximately 1.2 x 10^7 bp in size and has an estimated %G+C content of 49. Pulsed-field gel electrophoresis combined with gene-specific hybridization indicates that there are five linkage groups (chromosomes). Current evidence suggests trophozoites are polyploid with approximately four linkage groups in the G1 phase of replication, and eight linkage groups in G2 (Adam, 2001).

The *G. lamblia* genome has linear chromosomes bordered by telomeres that are akin to those in other eukaryotes (TAGGG) (Adam, 1991; Adam, 2001; Le Blancq *et al.*, 1991). *Giardia lamblia* has all four of the core histones that are used in eukaryotes to form chromatin from chromosomal DNA. These histones do not share similarity to equivalent proteins in the Archaea (Adam, 2001; Wu *et al.*, 2000).

Certain aspects of transcription in *G. lamblia* are more similar to eukaryotes than to prokaryotes, however, some features are more typically found in prokaryotes. *Giardia lamblia* transcripts are transported into the cytoplasm from the nucleus, where they are produced. Transcripts are polyadenylated, which is also typical of eukaryotes. However, there are short 5' untranslated regions and a lack of introns, both of which are more typical of prokaryotes, though introns are infrequent in unicellular eukaryotes (Adam, 2001).

1.6 Modes of Transmission

Giardia lamblia is found worldwide in a number of mammals. From 1992 to 1997 surveillance in 43 states demonstrated that there are as many as 2.5 million cases of giardiasis per year (Furness *et al.*, 2000).

Giardia lamblia cysts can be detected in raw and chlorinated community drinking water supplies, and in a study in British Columbia, Canada, 64% of raw water samples tested positive. In this same study, cyst viability was tested by infectivity in Mongolian gerbils (*Meriones unguiculatus*) and it was found that viability decreased after reservoir settling and chlorination (Isaac-Renton *et al.*, 1996).

Another Canadian study of the prevalence of *G. lamblia* in treated drinking water demonstrated that 18.2% of samples were positive for cysts (Wallis *et al.*, 1996). *Giardia lamblia* presents a risk to water supplies because it is more resistant to chlorine than bacteria. Cysts can be removed by filtration (Dawson, 2005).

Combined sewer overflows (CSOs) are discharges of mixed untreated sewage and stormwater that are released directly into receiving waters during rainfall. CSOs accommodate hydraulic strain when combined flows exceed system capacity. A study of CSOs determined that they are a significant contributor of *G. lamblia* to surface waters (Arnone & Walling, 2006). *Giardia lamblia* cysts have an average cyst density of 1013 kg m^{-3} and a settling velocity of 0.67 μm s^{-1}. The latter value indicates cysts settle extremely slowly out of suspension, highlighting the potential for *G. lamblia* infection from surface waters (Dai & Boll, 2006). It is estimated that cysts can remain infectious for more than 77 days at temperatures lower than 10°C (Rooney *et al.*, 2004).

Foodborne-outbreaks of *G. lamblia* are typically smaller in number of people infected than water-borne and are typically traced to fresh foods that are hard to thoroughly clean (Dawson, 2005).

1.7 Epidemiology

Giardia lamblia is transmitted via the fecal-oral route. Cysts can be transmitted to a new host via fecally contaminated food, water, or direct contact.

A controversial issue within the epidemiology of this parasite is whether or not it is zoonotic (Bauer, 1994; Bemrick, 1988; Connaughton, 1989; Faubert, 1988; Monis & Thompson, 2003; Thompson, 1998). This topic has been hotly debated for many years, with some authors concluding that there is evidence supporting that it is zoonotically transmitted from dogs (Traub *et al.*, 2004), while other authors generally *suggest* that it is zoonotic (van Keulen *et al.*, 2002). A recent review article on the topic of *G. lamblia* zoonosis concluded that in general there is not evidence to support that there is a major risk of zoonotic transmission to humans (Hunter & Thompson, 2005). To emphasize the lack of advancement on this topic, one must merely refer to a paper discussing the possibility of *G. lamblia* being zoonotic published in 1988, in which Faubert concludes that *G. lamblia* is both a zoonosis and a zooanthroponose (Faubert, 1988). Faubert's conclusion on the topic of zoonotic potential is significant because it suggests that human genotypes of *G. lamblia* are primarily going from humans to animals, not vice versa. Indeed, when looked upon from this perspective, previous studies and the study results

outlined in Appendix A of this work indicate that *G. lamblia* is indeed anthroponotically transmitted—that is, animals are most likely infected by humans with human genotypes.

There have been studies in which dogs living in close proximity to owners have harbored *G. lamblia.* The advent of molecular typing tools led to genotyping samples from these humans and dogs with interesting results. In an Aboriginal community in rural Australia dogs harbored dog genotypes (Hopkins *et al.*, 1997).

Other researchers believe that *G. lamblia* presents a risk of zoonosis—from dogs (Eligio-Garcia *et al.*, 2005; Ponce-Macotela *et al.*, 2005), cattle (O'Handley *et al.*, 2000), and farm and wild animals (van Keulen *et al.*, 2002).

1.8 Detection and Molecular Developments

Traditional *G. lamblia* detection in a human or animal involves microscopic examination of at least three concentrated stool samples. Simple microscopy was improved upon by the development of specific fluorescent antibodies that bind to *G. lamblia* cell wall proteins and clearly illuminate the presence of cysts in a sample by the use of a fluorescent microscope (Sterling, 1988). These fluorescent antibodies were developed into a commercially available direct immunofluorescence assay, which when evaluated against traditional ova and parasite examination was shown to be superior (Garcia *et al.*, 1992). When this kit was compared commercially available ELISA kit, it was shown to be a comparable method for detecting *G. lamblia* cysts in stool (Zimmerman & Needham, 1995). In fact, in one study it was used as a standard to

compare the effectiveness of different commercial ELISA kits to detect *G. lamblia* infection (Weitzel *et al.*, 2006).

Traditional polymerase chain reaction (PCR) of the small subunit ribosomal DNA (SSU-rDNA) has demonstrated greater sensitivity than microscopy in detecting feline *G. lamblia* infections (McGlade *et al.*, 2003).

1.9 Molecular Typing within Genotypes

The advent of cloning, PCR, RFLP and sequencing has lead to a large accumulation of *G. lamblia* genotype information. As previously mentioned, A-G are generally accepted genotypes of *G. lamblia.* For the purposes of tracing the transmission of *G. lamblia* within families and communities, molecular types have been found within the A and B genotypes by single nucleotide polymorphisms (snps).

Sequence information from SSU-rDNA sequences and various housekeeping genes have been used to define genotypes of *G. lamblia.* SSU-rDNA is the most commonly used genotyping tool for *G. lamblia*. Primers for PCR were first developed to SSU-rDNA sequences for genotyping purposes in 1992 (Weiss *et al.*, 1992). The most commonly used primer set for rDNA genotyping amplifies the 5' end of the 16S SSU and incorporates a small region of intergenic space that has more heterogeneity making it useful for genotype discrimination (Hopkins *et al.*, 1997); it has been used to genotype *G. lamblia* from dairy calves (O'Handley *et al.*, 2000), domestic cats (McGlade *et al.*, 2003), children and dogs (Eligio-Garcia *et al.*, 2005), and has been used in combination

with new genotyping techniques with housekeeping genes (Abe *et al.*, 2005; Caccio *et al.*, 2002; Lalle *et al.*, 2005).

Developing genotyping techniques with housekeeping genes is critical because they have more heterogeneity in their sequences, which allows for molecular typing within the genotypes. The high level of conservation within the rDNA sequences of *G. lamblia* is a limitation of the genotyping technique because it does not allow molecular typing within genotypes. A sequence-based approach using the *triose phosphate isomerase* (*tim* or *tpi*) gene was found to be useful—there was 18% nucleotide divergence between the sequenced A1 isolate (WB) and a B isolate (GS), and a 1% nucleotide divergence between WB and an A2 isolate (JH) (Baruch, 1996). More recently, 12 molecular types of the B genotype were identified by PCR and sequencing of a select region within the *tpi* gene (Sulaiman *et al.*, 2003). A wastewater study in Milwaukee using the same primers yielded five molecular types of genotype A, and eight more molecular types of B (Sulaiman *et al.*, 2004).

The *β-giardin* (Caccio *et al.*, 2002; Lalle *et al.*, 2005) and *glutamate dehydrogenase* (*gdh*) (Abe *et al.*, 2003; Abe *et al.*, 2005; Read *et al.*, 2004) genes have also been used for molecular typing.

1.10 Evolutionary Questions

The complexity of our understanding of *G. lamblia* is not limited to its taxonomy or biology, but an intertwining of the two. This organism was thought to be the most primitive eukaryote because it lacks certain organelles, including, the mitochondrion

(Adam, 2001). However, Tovar *et al* claimed to have found mitochondrial remnant organelles that put the early evolutionary status of *G. lamblia* into question (Tovar *et al.*, 2003). However, it remains possible that Giardia is still an early diverging eukaryote, although probably not pre-mitochonriate.

Sexual replication has never been documented, and *G. lamblia* has long been assumed to replicate asexually or clonally (Tibayrenc *et al.*, 1990). Most of the population genetic evidence to date has supported clonal replication. It has frequently been considered as a primitively asexual organism. However, a recent publication by Ramesh *et al* indicates that *G. lamblia* has the genes that are necessary for meiotic replication in other organisms (Ramesh *et al.*, 2005).

1.11 Disease and Pathogenesis

Giardia lamblia was not identified as a disease-causing parasite until more than 50 years ago (Boreham *et al.*, 1990). The incubation period may range from 12 to 19 days, which is followed by an acute phase where symptomology may emerge (Faubert, 2000; Jokipii *et al.*, 1985).

Symptoms of *G. lamblia* can be, but are not limited to, diarrhea with no blood or mucus in the stool, flatulence, abdominal pain, belching, malabsorption and failure to thrive or poor weight gain (Nash, 2001). The symptoms of *G. lamblia* are most likely caused by the trophozoites attaching to the microvilli, causing ultrastructural changes such as shortening and disruption of said structures (Farthing, 1997).

Giardia lamblia infection is not always accompanied by symptoms, as many individuals are asymptomatic and are thus hidden carriers of the parasite. There is evidence that asymptomatic giardiasis is associated with detrimental effects in cognition in children in developing countries, as they are usually not treated (Berkman *et al.*, 2002). A study performed in Peruvian children ages one to three demonstrated that 32% of children (46/143) with diarrhea caused by *Cryptosporidium parvum* and *G. lamblia* were growth stunted. Severe growth stunting in the second year of life led to children scoring 10 points lower on a cognitive test when compared to children without severe stunting. In children with more than one episode of *G. lamblia* per year, cognitive tests were 0.2-8.0 points lower than children with one or less episodes per year. Statistical analysis demonstrated that neither the prevalence of diarrhea nor infection with *C. parvum* was associated with lower cognitive test scores—possibly meaning that *G. lamblia* was associated with the lowering of cognition (Berkman *et al.*, 2002). A study in Brazil supports the argument for treating asymptomatic giardiasis in children (Prado *et al.*, 2005).

1.12 Immune Response

Information about host immune responses to *G. lamblia* has focused on studies on neonatal mice, or assessment of the analogous parasite in the murine model using *G. muris* (Scott *et al.*, 2004). In one study, transgenic mice lacking IgA-expressing B-cells, IgM-secreting B-cell, or all B cells were infected with *G. lamblia* and *G. muris* where

IgA antibodies were found to play a central role in clearing the infection (Langford *et al.*, 2002).

In mice, *G. muris* infection was shown to cause damage in intestinal mucosa via the reduced ability of cells to exclude tryptan blue and reduced levels of brush border lactase, sucrase and maltase (Anand *et al.*, 1985).

Some studies of murine giardiasis have suggested that host factors may be responsible for some of the intestinal damage from giardiasis. Athymic mice cannot clear a *Giardia* infection according to two studies (Heyworth *et al.*, 1985; Roberts-Thomson & Mitchell, 1978). To assess whether $CD4^+$ or $CD8^+$ T lymphocytes may play a role in intestinal injury, mice were given purified lymphocytes from mice previously infected with *G. muris.* It was found that $CD8^+$ T cells, but not $CD4^+$ T cells caused brush border injury (Scott *et al.*, 2004).

Unfortunately for the host, *G. lamblia* trophozoites have an arsenal of variant surface proteins (VSPs) that can be expressed to avoid the host antibody response—it is estimated that there are 300 VSP genes, which are cysteine rich and make up nearly 4% of the genome (Adam, 2001; Nash & Mowatt, 1992; Singer *et al.*, 2001; Smith *et al.*, 1998). The switch from one VSP to another is referred to as antigenic variation (Adam, 2001; Adam *et al.*, 1988; Aggarwal & Nash, 1988; Nash *et al.*, 1988).

1.13 Treatment

Giardia lamblia can be eliminated from the host by treating with one of several antimicrobial agents with activity against *Giardia* spp. Quinacrine was initially the most widespread drug used in the United States but is no longer routinely available. Thus, metronidazole is the most commonly drug used for treating human giardiasis. More recently, another nitroimidazole, tinidazole, was approved for this purpose by the Food and Drug Administration in the United States (Fung & Doan, 2005). Albendazole has also been efficacious as an alternative therapy for giardiasis (Yereli *et al.*, 2004). Furazolidone has been commonly used for treating giardiasis in children and nitazoxanide has also shown efficacy for treatment of giardiasis (Bailey & Erramouspe, 2004)

There are a small number of patients who experience treatment failure, and are referred to as having refractory giardiasis. Whether true drug resistance occurs is not yet known in part because of the difficulty of axenizing *G. lamblia* in order to perform susceptibility tests. Patients with refractory giardiasis are typically treated with longer courses and/or combinations of drugs such as quinacrine plus metronidazole.

Dogs and cats are treated for giardiasis largely with metronidazole or albendazole, though a recent study suggested that a febantel-praziquantel-pyrantel product may be an efficacious alternative treatment with less negative side-effects (Payne *et al.*, 2002).

2.0 PRESENT STUDY

This dissertation describes new molecular typing techniques that impact our understanding of *G. lamblia* transmission in families and communities. The first aim describes the molecular epidemiology of *G. lamblia* in an endemic community in Lima, Peru, where *G. lamblia* positive family members and canine companions were analyzed for genotype carriage. Potentially novel molecular types of the A2 genotype were described by extensive PCR and sequencing. The second aim resulted from the unexpected finding that different chromosome genotyping yielded different molecular type groupings for the same samples. This led to questions about the basic biology of the parasite: are these grouping differences due to sexual recombination? Algorithmic analysis was used to analyze the sequence data to determine the statistical probability that sexual recombination is occurring.

The methods, results, and conclusions of the two studies are presented in the appended manuscripts. The most important findings are summarized in the following sections.

2.1 Molecular typing of *G. lamblia*: a sequence-based approach

This manuscript outlines a multi-locus sequencing approach to molecular typing of samples of *G. lamblia* obtained from a Peruvian peri-urban shantytown. This community provided the ideal setting for investigating the patterns of genotype infection in an endemic region. The presence of an established public health team and clinic allowed for the sampling of multiple family members and family dogs. In this way it was possible to

understand and identify genotype infections between family members, and by genotype analysis of dog samples, to assess the possibility of zoonotic transmission occurring from dogs to human family members. Non-cloned PCR sequence data demonstrated that dogs, with one exception, harbored the dog-specific C and D genotypes. We propose that *G. lamblia* is anthroponotic—it is more likely that dogs are more in danger of being infected from humans than the opposite.

2.2 Unexpected findings in molecular typing of *G. lamblia:* evidence for sexual recombination

The second manuscript in this dissertation describes the unexpected finding of evidence that *G. lamblia* is undergoing sexual recombination. It was not possible to molecular subtype A2 genotype samples from the Peruvian peri-urban shantytown using published PCR and sequencing protocols. In order to detect sequence heterogeneity within the samples from the study site, sequencing was performed across broad areas of chromosomes 3 and 5 in order to find a region of heterogeneity that would be useful for a molecular subtyping tool. Interestingly, phylogenetic trees generated for the same samples at the two loci did not show the same groupings. This suggested the possibility of sexual recombination occurring. To confirm or dismiss this possibility, algorithmic analysis through the Geneconv program was used to analyze the sequence data. Results from the Geneconv analysis demonstrated regions of recombination, confirming that sexual recombination is occurring in A2 samples of *G. lamblia.*

3.0 RESEARCH CONTRIBUTION

By characterizing the molecular epidemiology of *G. lamblia* in a Peruvian peri-urban shantytown, I was able to contribute to a better understanding of the patterns of infection of this important microorganism.

This work describes the development a technique of molecular typing for the A2 genotype of *G. lamblia* that can be applied by the protozoology research community in order to better comprehend the patterns of transmission of this microorganism among family members.

An unexpected finding was that in designing a molecular typing of the A2 genotype, extensive sequencing and sequence comparison of A2 sample groupings on two chromosomes raised our suspicions that meiotic recombination may be occurring. The latter changes the paradigm of how *G. lamblia* molecular typing must be approached.

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APPENDIX A:

MULTILOCUS ANALYSIS OF *GIARDIA LAMBLIA* ISOLATES FROM A HIGHLY ENDEMIC REGION OF PERU

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Abstract.

Giardia lamblia is ubiquitous in developing areas where pure water is not readily available. It is now recognized that even asymptomatic infection may cause growth stunting and mental retardation in affected children; therefore it is important to prevent and control infections in these settings. Whether dogs are an important source of infection remains controversial. Nash groups 1, 2, and 3 (genotypes A1, A2, and B, respectively) are found in humans, while genotypes C and D are typically found in dogs. However "human" genotypes have occasionally been identified in dogs. The epidemiology of *G. lamblia* was assessed in humans and dogs in a shantytown of Lima, Peru. Human infections were all caused by isolates of genotypes A2 and B. With one exception, dogs harbored the C and D genotypes, suggesting that transmission between dogs and humans occurs rarely. We used a multilocus sequence-based approach to distinguish isolates within genotypes A2 and B. The snp analysis identified multiple subtypes within each genotype making it possible to more accurately trace individual isolates. Interestingly, a phylogenetic analysis of the loci from different chromosomes yielded different trees, a result that would be predicted if recombination occurs between different isolates. This observation is consistent with our other data suggesting that meiotic replication within the A2 genotype. These results suggest the importance of using multilocus typing and subtyping for accurate molecular characterization of potential transmission patterns.

Introduction.

Giardia lamblia (syn. *Giardia intestinalis, Giardia duodenalis*) is an intestinal protozoan parasite that is ubiquitous in mammals. It has two stages, a trophozoite and cyst stage, the latter being responsible for transmission. Pampas de San Juan de Miraflores, Peru, a peri-urban shantytown, is hyperendemic for *G. lamblia*. The majority of children in this shantytown have been found to be infected with this protozoan parasite by the age of three years (Gilman *et al.*, 1988). Infection with the parasite is associated with negative effects to the mental and physical development of children in their age cohort. This study revealed that children who suffered more than one case of diarrhea per year scored significantly lower on an intelligence quotient test (Berkman *et al.*, 2002). In Malaysia giardiasis was identified as a statistically significant predictor of childhood malnutrition (Al-Mekhlafi *et al.*, 2005). Asymptomatic giardiasis has been associated with physical stunting and cognitive deficiencies to children in developing countries (Checkley *et al.*, 2004; Checkley *et al.*, 1997).

A substantial degree of genetic heterogeneity has been identified among *G. lamblia* isolates from a variety of mammals. These have been divided into genotypes A through G on the basis of molecular sequence differences and reflect differences in host specificity and biology. The "human" genotypes were originally designated as Nash groups 1, 2, and 3 (genotypes A1, A2, and B, respectively) and are found primarily in humans (Nash & Keister, 1985; Nash *et al.*, 1985). Genotypes C and D are typically found in dogs and genotypes E-G are found in other mammals.

The SSU-rDNA sequence has been the most useful for genotype assignment (Hopkins *et al.*, 1997; Read *et al.*, 2002; Weiss *et al.*, 1992), but the rDNA sequence is too conserved to allow subtyping within genotypes.

Single nucleotide polymorphisms (snps) within protein coding genes has been quite useful for subtyping within genotypes A1, A2, and B. Several loci have been used at the sequence level to designate *G. lamblia* genotypes: *triose phosphate isomerase* (*tpi*) (Amar *et al.*, 2002; Baruch, 1996; Sulaiman *et al.*, 2003), *glutamate dehydrogenase gene* (*gdh*) (Read *et al.*, 2004), and *β-giardin* (Lalle *et al.*, 2005)

However, there are a number of exceptions to these generalizations. The only genotypes that have been found in humans are genotypes A1, A2, and B. However, these genotypes have occasionally been found in beavers, cats, dogs, and other mammals. For this reason, controversy remains over the possibility of zoonotic transmission of *G. lamblia*, (Faubert, 1988; Thompson, 2004). Therefore, it is critical to assess the risk of zoonotic transmission of so-called human genotypes of *G. lamblia* from a companion animal—the dog (Monis *et al.*, 2003; Thompson, 2004). A study performed in a rural Aboriginal community in Australia demonstrated that dogs in close proximity to humans under compromised hygienic conditions are most likely to harbor one or two "dog" genotypes of *G. lamblia*, designated as C or D. Most significantly none of the dogs harbored genotypes that have been found to infect humans. Interestingly, dogs in urban settings in Australia were found to have "human genotypes" (Hopkins *et al.*, 1997). A study of tea plantation communities in India demonstrated that dogs often harbored human genotypes of *G. lamblia* (Traub et al., 2004).

Materials and Methods.

Study Site.

Pampas de San Juan de Miraflores is a desert shantytown 25 km to the south of Lima, Peru. The last census recorded the population at 38,721 in April of 1997. Inhabitants are mainly mestizo migrants from the Peruvian Andes Mountains who earn a living by performing unregulated day labor (Johnson *et al.*, 2004). More details about the demographics and environment of this shantytown have been described in previous literature (Bern *et al.*, 2002; Checkley *et al.*, 1998; Checkley *et al.*, 2002).

Patient Identification. Initially, children who tested positive for *G. lamblia* by stool microscopy within a *Cyclospora cayetanensis* study were enrolled in this study. Once a child tested positive, as many other household members as possible were enrolled within this study. In this way many individuals within a family were enrolled and gave invaluable data for the better understanding of the molecular epidemiology of *G. lamblia* among family members. All adult patients enrolled in the study signed written consent forms, and parents signed written consent forms for children and infants. The Institutional Review Board of PRISMA and the Human Subjects Approval Committee at the University of Arizona approved the protocol used for the study.

Patient sampling. Bi-weekly stool samples were screened microscopically for *G. lamblia* cysts, and positive samples were prepared for DNA analysis.

Canine sampling. Canine fecal samples were obtained from families enrolled in the study. Dogs in the shantytown are largely free roaming with few exceptions, thus dogs were given suppositories and visibly monitored for defecation. Canine fecal samples were collected and monitored microscopically for *G. lamblia* cysts. Positive samples were prepared for DNA analysis.

Preparation for DNA analysis. Fecal samples were purified by sedimentation using methods already described (Melvin *et al.*, 1982) with the exception that purified double distilled water was used in lieu of formalin. Two hundred microliters of fecal concentrate were purified via the QIAmp DNA Stool Kit (QIAgen, Valencia, CA).

Molecular typing of fecal *G. lamblia* samples. A nested *tpi* PCR (Sulaiman *et al.*, 2003) was used to distinguish A from B samples. Two µl of purified DNA from human fecal samples were used as template. Non-acetylated bovine serum albumin (20mg/ml) at a final concentration of 0.5% and dimethyl sulfoxide (DMSO) at a final concentration of 10% were used to optimize DNA amplification. Magnesium chloride concentration, *tpi* primer concentration (300µM external primers, 200µM nested primers) and PCR amplification conditions were implemented in a 50-µl reaction under reaction conditions as previously described (Sulaiman *et al.*, 2003). External and nested PCR products were visualized using agarose gel electrophoresis. Nested PCR products were purified using the microcentrifuge based Gel Extraction protocol of the QIAquick PCR purification kit

(QIAgen, Valencia, CA). Sequencing was performed on nested PCR products using nested amplification primers with the addition of DMSO.

A nested *tpi* PCR (Amar *et al.*, 2002) was used to detect mixed A and B genotypes, as the above protocol was not designed to detect mixed infections. Samples were genotyped by nested PCR as described (Amar *et al.*, 2002).

Small sub-unit ribosomal RNA (SSU-rRNA) PCR. Two μl of purified DNA from canine fecal samples were used as template. Non-acetylated bovine serum albumin (20mg/ml) at a final concentration of 0.5% and dimethyl sulfoxide (DMSO) at a final concentration of 10% were used to optimize DNA amplification. Magnesium chloride concentration, SSU-rRNA primer concentration, and PCR amplification conditions were implemented in a 50-μl reaction under reaction conditions as previously described (Hopkins *et al.*, 1997). A previously described nested PCR was used to increase the yield and sensitivity (Read *et al.*, 2002). External and nested PCR products were visualized using agarose gel electrophoresis. Nested PCR products were purified using the microcentrifuge based Gel Extraction protocol of the QIAquick PCR purification kit (QIAgen, Valencia, CA). Sequencing was performed on nested PCR products using nested amplification primers with the addition of DMSO.

A2 subtyping. Two nested PCR reactions were designed—one on chromosome 5 and another on chromosome 3. Primer pair 12F/12R (Table 2) was used for amplification of the external product and the primer pair 12NF/12NR (Table 2) was used for nested PCR

using the 12F/12R primer amplification product as template. The 732 bp nested PCR product was then purified and sequenced as previously described in this paper. The second nested PCR was designed for A2 molecular typing for chromosome 3 and performed in an identical manner except that external (1F/15R) and nested (15F/15R) were used specific to that locus (Table 2) and produced a 526 bp nested PCR product. Concentrations of PCR master mix ingredients were as follows: 1.5mM magnesium chloride, 1μM primers, and 1μM dNTPs. To improve yield, PCR master mixes included 0.5% non-acetylated bovine serum albumin (Sigma) and 10% dimethyl sulfoxide (Sigma). Thermocycler conditions were as follows: 2 minute initial denaturing (94ºC), 40 cycles of 30 seconds denaturing (94ºC), 30 seconds annealing (51ºC), 1 minute extension (72ºC), followed by 10 minutes of final extension (72ºC). Samples were purified and sequenced as described previously in this manuscript.

B subtyping. A nested PCR reaction was designed on chromosome 3 to complement the previously published (Sulaiman *et al.*, 2003) primer set on chromosome 5. Primer pair 2899-168F/2798-422R (Table 2) was used for amplification of the external product and the primer pair 2899-168F/2798-660R (Table 2) was used for nested PCR using the 2899-168F/2798-422R primer amplification product as template. The 772 bp nested PCR product was then purified and sequenced as previously described in this paper. PCR master mix components were added at the same concentrations as described above. To improve yield, BSA and DMSO as described in the previous paragraphs were added. Thermocycler conditions were as described in the previous paragraph. Samples were

purified and sequenced as described previously in this manuscript.

Results.

Genotype assignment of *G. lamblia* isolates. Human and canine stool samples were screened, and *G. lamblia* positive samples were assessed for molecular genotype and subtype (Table 1). Most of the studies distinguishing between *G. lamblia* genotypes have been based on differences in SSU-rDNA sequence. This approach allows differentiation of the human genotypes A and B as well as the dog genotypes C and D (Hopkins *et al.*, 1997; Read *et al.*, 2004). A nested PCR within this SSU-rDNA region allows for greater sensitivity and yield for samples that do not amplify with the initial primer set (Read *et al.*, 2002). This approach was used to determine the genotypes of the human and dog isolates in the study population. The C and D genotypes that have been published (Thompson *et al.*, 2000) are identical in dogs in this region to samples collected in dogs in Peru and Arizona. Sixty-four of the 84 *G. lamblia*-positive canine stool samples were successfully genotyped. These 64 samples represented 49 dogs. All genotyping on canine samples using SSU-rDNA sequencing yielded dog genotypes: 29 dogs had the D dog genotype and 18 dogs had the C dog genotype. One dog had a C then C/D mixed infection, a second dog had a D then C/D mixed infection, and a third dog had a single C/D mixed infection. Mixed genotype infections were detected via a double peak in the chromatogram.

Identification of mixed A2 and B infections. A limitation of the SSU-rDNA genotyping technique is that it does not allow for detection of mixed genotypes, unless they are present in nearly equal qualities. For the purpose of mixed genotype detection, we used a

heteroduplex PCR which amplifies different sizes of products from A2 or B genotypes at the *tpi* gene (Amar *et al.*, 2002).

Analysis of B genotypes of *G. lamblia* using a published set of nested PCR primers allowed for distinguishing between different subtypes (Sulaiman *et al.*, 2003) However, a difficulty encountered with this method was that mixed genotype infections were difficult, if not impossible to detect via this method. A PCR method that allows for mixed genotype detection is a heteroduplex approach (Amar *et al.*, 2002). With this method it was possible to identify mixed genotype infections, and additionally, a restriction fragment length polymorphism (RFLP) reaction of a nested product allowed for distinguishing between the A1 and A2 genotype. A difficulty encountered in this procedure was that sequencing of the Amar nested product did not yield any identifiable nucleotide polymorphisms to allow for distinguishing between isolates.

One family analyzed by this technique family had a dog with a *G. lamblia* infection, where samples from this canine were tested via the latter method (Amar *et al.*, 2002), and it was discovered that the dog had a B genotype infection. Interestingly, the dog simultaneously had a dog genotype D infection—mixed A/C genotypes in dogs have been reported previously (Berrilli *et al.*, 2004). This household demonstrated both stable and changing patterns of *G. lamblia* infection that could be detected over the sampling period of the two years (Table 3).

Molecular typing classification of Genotype A2 isolates. A2 positive samples were amplified using the novel chromosome 5 and chromosome 3 primers (Table 2) that were developed for molecular subtyping. The primers were developed through extensive

nucleotide polymorphism analysis of five Peruvian A2 genotype *G. lamblia* isolates. The analysis included 10 kb of sequencing on chromosome 5 and 6 kb on chromosome 3 (manuscript in preparation). The most heterogeneous region on chromosome 5 was 900 bp at the distal end of the sequenced region. A nested PCR method, yielding a 732 bp product, was chosen to increase sensitivity and yield for sequencing to overcome the potential difficulty of amplifying stool matrix isolates. For the 6 kb of chromosome 3, snp analysis revealed that the proximal end was best for subtyping. A nested PCR yielding a 526 bp product was developed.

The nested PCR reactions that were developed allowed for distinguishing between molecular types of A2. This allowed for tracing molecular types of A2 within the Peruvian periurban shantytown in order to better understand the dynamics of transmission of this parasite.

Molecular subtypes were established from phylogenetic trees generated from sequence data for chromosome 3 (Figure 1A) and chromosome 5 (Figure 1B). Molecular types for A2 at chromosome 3 were labeled 3A-I to 3A-VII, and at chromosome 5, they were labeled 5A-I to 5A-VI. Field isolates were categorized by 3A and 5A molecular type in tabular form (Table 3).

Molecular typing classification of Genotype B isolates. Genotype B isolates were typed at the *tpi* gene using primers that have been described (Sulaiman *et al.*, 2003; Sulaiman *et al.*, 2004). Seven novel molecular types of the B genotype were identified in the Peruvian shantytown, and were designated BS-13 to BS-19 (Figure 2A). A sequence-

based approach was developed at a second locus adjacent to the *γ-giardin* gene on chromosome 3 for B molecular typing purposes (Figure 2B) and 12 molecular types were identified. The molecular types for B samples at the chromosome 3 locus were labeled 3B-I to 3B-XII. Field isolates were categorized by 3B molecular type in tabular form (Table 5).

The molecular typing method for B samples on chromosome 3 allowed for a multilocus approach that was in the same region as the chromosome 3 A2 molecular typing approach described in the following paragraph.

Discussion.

Giardia lamblia was recently added to the World Health Organization's (WHO) "Neglected Diseases Initiative," highlighting the need to control this parasite in poverty-stricken areas of the developing world (Savioli *et al.*, 2006). In contrast, infections of this parasite in people of developed nations usually have limited long-term effects, with the exception being those individuals afflicted with refractory giardiasis (Nash *et al.*, 2001).

In order to comply with the WHO initiative (Savioli *et al.*, 2006), it is critical to develop tools to trace the molecular epidemiology of *G. lamblia* infections in endemic communities. The current study describes the use of a multilocus sequence-based approach for molecular typing *G. lamblia* samples. This is critical for hyperendemic communities in order to better understand the epidemiology of the parasite at the molecular level.

Genotyping has been critical for understanding the zoonotic potential of giardiasis from dogs. The current study demonstrated the rarity of zoonotic transmission of giardiasis in a region that is endemic for canine and human giardiasis. All dogs with one exception had C, D, or mixed C/D, except for one mixed B/D. Genotype C and D have not been reported in humans, and it is likely that the one B sample in the dog was anthroponotic. Dogs in the Peruvian shantytown appear to be circulating dog genotypes, which is similar to the results found in Australia. In an Aboriginal community in rural Australia dogs harbored dog genotypes (Hopkins *et al.*, 1997). The single dog in the current study, which had the B genotype, simultaneously harbored a D genotype

infection. In the current study, it was found that a child in the family, 95-F had a B genotype at nearly the same time. The presence of a mixed B/D infection followed by a D and then C genotype infection in the family dog suggests that the dog primarily and preferentially hosted C and D genotype infections. Recent phylogenetic analysis of genotypes of *G. lamblia* found in humans and animals suggests species-specificity (van der Giessen *et al.*, 2006), which is in agreement with what was found in this study in dogs. The Australian and Peruvian studies both support the notion of occasional anthroponotic transmission. Conversely, the risk of zoonotic transmission appears to be quite low.

The multilocus molecular typing approach used in this study represents a more complete method of assessing the molecular epidemiology of field cases of giardiasis. Molecular typing data from the current study yielded different molecular type groupings of the same samples at different loci. The latter has been observed before (Robertson, 2006). In another study, subtyping of field samples at the *elongation factor-alpha* (*elf-α*) gene demonstrated "poor genetic resolution" when compared to established genotyping/subtyping approaches (Traub *et al.*, 2004). From the conflicting data obtained, it was recommended that a multilocus approach be taken for subtyping assessment of *G. lamblia* samples; this would seemingly resolve the issue of "poor genetic resolution" (Traub *et al.*, 2004). The current study uses a multilocus approach, and takes molecular typing assessment to the level of different chromosomes. It is possible that the seemingly conflicting data in this study and others (Robertson, 2006; Traub *et al.*, 2004) may be attributed to meiotic or other recombination. Evidence for

meiotic or other recombination has been uncovered (manuscript in preparation) in several field samples from this study.

Molecular epidemiologists must take note of the importance of taking a broader approach to molecular typing, as in the current study, regardless of whether or not meiotic or other recombination is directly observed in *G. lamblia*. It is only by fully understanding the biology of this complex microorganism that we may properly and successfully address the "Neglected Disease Initiative" of the WHO (Savioli *et al.*, 2006), and work to improve the lives of children in developing nations.

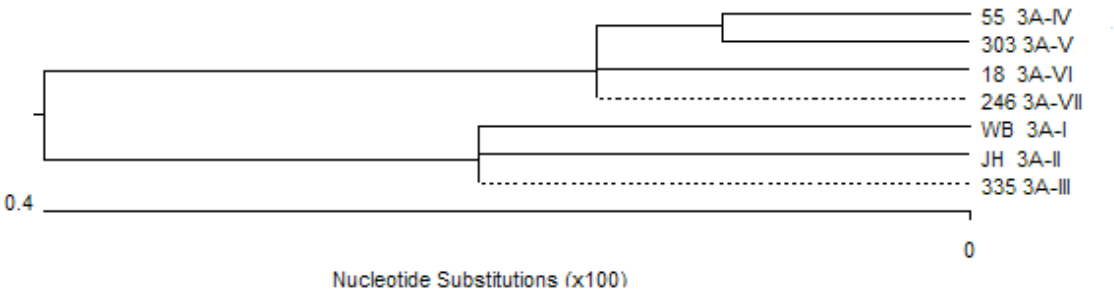
Table 1. Data set analyzed

Sample Year	2002	2003
Total human samples	778	753
Positive human samples	159 (20.4%)	154 (20.5%)
Total dog samples	25	580
Positive human samples	4 (16%)	84 (14.5%)

Table 2. Primers (underlining denotes nested primer); *denotes published primers (Sulaiman et al, 2003).

Genotype Assessed	Chromosome locus	Primer 1	Primer 2
A2	adjacent to *γ-giardin* on chromosome 3	3c-1F TGGAGGCGGTCAAGATACTC 3c-1.5F CGGTCAAGATACTCTACGATCG	3c-1.5R CTCGACGATTATGCTCCACGACG 3c-1.5R CTCGACGATTATGCTCCACGACG
A2	adjacent to *tpi* on chromosome 5	5c-12F GGCGAGTGCAGTCCTGAGTGG 5c-12NF CAGTTTGGAAGAGCAGGACTCG	5c-12R CCTGGCTTGTTAACTACATCC 5c-12NR GTCATCTTTCTATGCCTTCTTCG
B	adjacent to *γ-giardin* on chromosome 3	2899-168F CTTCTCGTTCATGCCACTGATGACG 2899-168F (same as external) CTTCTCGTTCATGCCACTGATGACG	2798-422R GTTGAGACAGAGAAATGTATCCAACG 2798-660R CTTGTACTTATGTGTGGAAAGTTCG
B	*tpi* coding region on chromosome 3	AL3543F* AL3544F*	AL3546R* AL3545R*

A.



B.

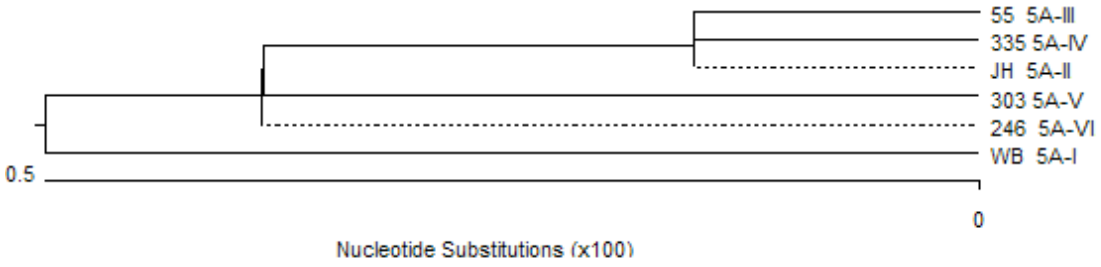
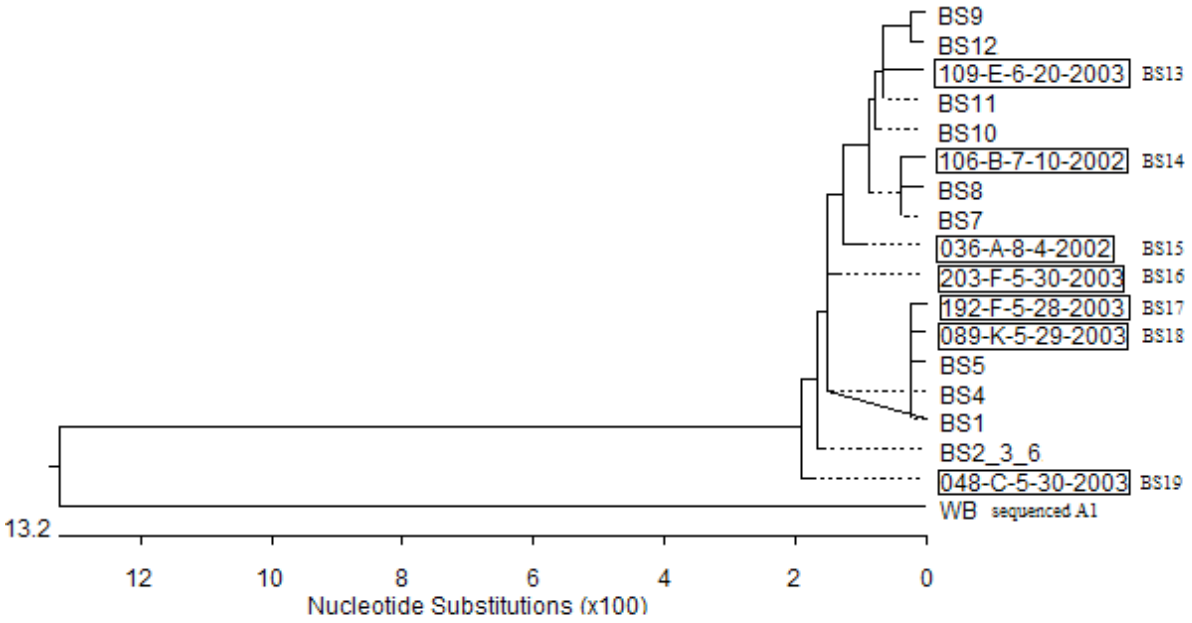


Figure 1. A. Molecular types of A *G. lamblia* genotypes on chromosome 3 adjacent to the *γ-giardin* gene. B. Molecular types of A *G. lamblia* genotypes on chromosome 5 adjacent to the *tpi* gene.

A.



B.

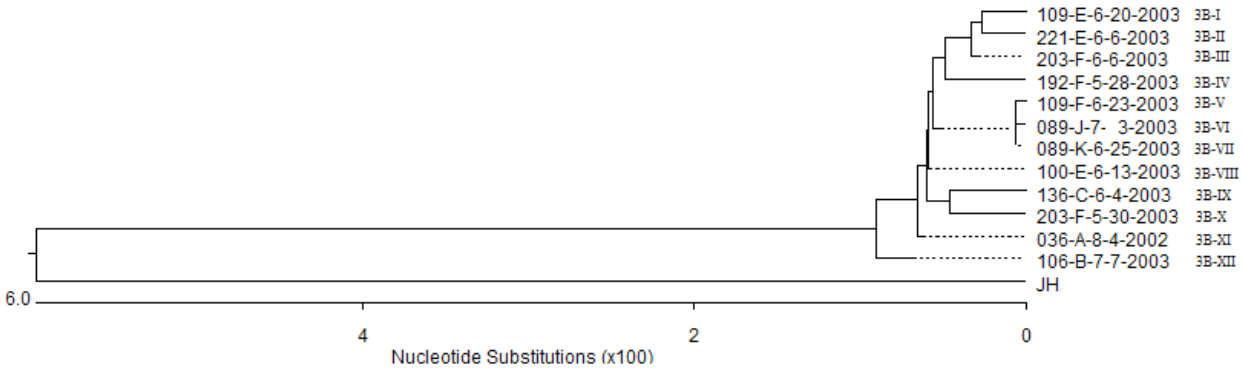


Figure 2. A. Molecular types of B *G. lamblia* genotypes on chromosome 5 in the *triose phosphate isomerase* (*tpi*) gene coding region. BS1-BS12 (Sulaiman *et al.*, 2003), BS13-BS19 new molecular types of B, and denoted in boxes (current manuscript).

B. Molecular types of B *G. lamblia* genotypes on chromosome 3 adjacent to the *γ-giardin* gene.

Table 3. Mixed genotypes and a dog with B infection in a single family (family 95).

	WINTER 2002							WINTER 2003								
Sibling	7/2	7/5	7/8	7/10	7/11	7/17	8/3	5/28	5/30	6/4	6/12	6/13	6/18	6/25	7/3	7/9	7/10
C	A2		B	B		B										A2	
D	A2/B	A2		A2		B		A2		A2	A2						A2
E																	A2
F		A2			A2		A2		B	B	B			B			B
Dog												B/D	D		C		

Table 4. A2 molecular types for human samples at two loci – 3A = chromosome 3 A genotype molecular types, 5A = chromosome 5 A genotype molecular types. Numerical molecular type designations correspond to groupings in phylogenetic tree in Figures 2A and 2B.

Family - Individual	Date processed	3A type	5A type
020-F	7/1/2002	IV	III
020-F	7/8/2002	VII	III
020-F	7/18/2002	VII	III
020-F	8/4/2002		II
020-F	5/30/2003	VII	II
020-F	6/5/2003	VII	II
058-C	7/12/2002		VI
058-D	7/1/2002		III
061-B	6/28/2002	VII	III
061-B	5/30/2003	V	II
061-B	6/30/2003		II
061-C	7/12/2002	V	IV
066-J	7/10/2002	VII	VI
082-D	5/28/2003	III	V
089-E	6/26/2003	VII	II
095-C	7/2/2002	IV	III
095-C	7/9/2003	IV	III
095-D	7/2/2002	V	IV
095-D	7/5/2002	IV	III
095-D	7/10/2002	IV	III
095-D	5/28/2003	IV	III
095-D	6/4/2003	IV	III
095-D	6/12/2003	IV	III
095-D	7/10/2003	IV	III
095-E	7/10/2003	IV	
095-F	7/2/2002	IV	III
095-F	7/5/2002	IV	III
095-F	7/11/2002	IV	III
095-F	8/3/2002	IV	III
097-H	5/29/2003		V
100-C	7/17/2002	III	
100-C	6/12/2003	III	
100-C	6/13/2003	III	III
100-C	9/30/2003	III	IV
100-D	6/19/2003	III	IV
100-D	9/25/2003	III	IV
100-D	9/27/2003	III	IV
100-E	9/24/2003	III	IV
100-E	9/27/2003	III	IV

100-E	10/2/2003	III	IV
100-E	10/9/2003	III	IV
100-E	10/14/2003	III	IV
100-E	10/20/2003	III	IV
100-F	6/4/2003	III	IV
100-F	6/12/2003	III	IV
100-F	6/13/2003	III	II
100-F	6/20/2003	III	IV
100-F	6/27/2003	III	IV
100-F	9/27/2003	III	IV
100-F	9/30/2003	III	IV
100-F	10/2/2003	III	IV
100-F	10/10/2003	III	IV
100-F	10/14/2003	III	IV
104-E	5/28/2003	III	II
104-F	6/19/2003	V	V
104-H	5/30/2003	V	V
104-H	6/5/2003	V	V
104-H	6/20/2003	V	V
104-H	6/25/2003	V	V
104-H	6/27/2003	V	V
104-I	5/28/2003	V	V
104-J	5/28/2003	V	V
104-J	6/12/2003	V	V
104-J	6/26/2003	V	V
104-J	7/9/2003	V	V
105-D	7/12/2002	VII	II
105-D	7/19/2002	VII	II
105-D	8/4/2002	VII	II
105-E	8/2/2002	VII	II
106-B	7/3/2002		IV
106-C	6/27/2002	III	IV
106-C	7/18/2002	III	IV
106-E	7/4/2003	III	IV
109-E	8/2/2002		III
110-F	5/29/2003	III	II
113-D	5/28/2003	III	
113-H	7/12/2002	V	VI
130-D	5/30/2003	III	
130-D	6/5/2003		VI
136-D	5/30/2003	III	III
136-D	6/4/2003	III	III
142-F	6/6/2003	VII	V
143-E	7/4/2003	III	
192-C	7/5/2002	VII	III
192-E	7/3/2002	VII	IV
192-E	8/2/2002	VII	
227-F	5/29/2003		V
227-F	6/4/2003		V
233-E	5/29/2003	III	IV

Table 5. B molecular types for human samples at two loci – 3B = chromosome 3 B genotype molecular types, 5B = chromosome 5 B genotype molecular types. Numerical molecular type designations correspond to groupings in phylogenetic tree in Figures 1A and 1B.

Family - Individual	Date processed	3B type	5B type
007-D	7/18/2002	VII	
036-A	8/4/2002		BS15
048-C	5/30/2003		BS19
089-J	7/3/2003	VI	
089-K	5/29/2003		BS18
089-K	6/25/2003	VII	
089-K	7/4/2003	VII	
100-E	6/12/2003	VIII	
100-E	6/13/2003	VIII	
100-F	5/29/2003	VIII	
106-B	7/10/2002		BS14
106-B	7/7/2003	XII	
109-E	6/20/2003	I	BS13
109-F	6/23/2003	V	
113-E	6/5/2003	VIII	
118-D	7/12/2002	VII	
136-C	6/4/2003	IX	BS6
142-B	7/17/2002	VIII	
143-F	7/8/2003	VII	
192-F	5/28/2003	IV	BS17
203-F	5/30/2003	X	BS16
203-F	6/6/2003	III	
214-E	7/18/2002	VIII	
221-E	6/6/2003	II	

Acknowledgments. The author would like to thank Drs. B. Helen Jost, Donna Wolk, and Lynn Joens for helpful commentary. Lilia Cabrera was critical in sample collection at the study site. This study would not be possible without the assistance of P.R.I.S.M.A. and funding from National Institutes of Health Minority International Research Training grant TW00036.

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APPENDIX B:

GENETIC EVIDENCE FOR RECOMBINATION FROM FIELD ISOLATES OF *GIARDIA LAMBLIA*

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Abstract.

Giardia lamblia is a eukaryotic parasite long assumed to be primitively asexual. However, in the absence of sexual reproduction, it is difficult to explain the remarkably low level of allelic sequence heterozygosity. In fact, the two nuclei are so similar that there must be some type of homogenization event to maintain their similarity. All *G. lamblia* isolates obtained from humans belong to Nash Groups 1, 2, and 3 (Genotypes A1, A2, and B, respectively). As part of an epidemiologic investigation of giardiasis within a Lima Peru shanty town, a sequence-based classification system was developed using 17 kb from four loci on three different chromosomes of the JH A2 isolate. In order to identify single nucleotide polymorphisms (snps) in A2 isolates, these same loci were sequenced for five field isolates from four different households. Snps were identified at all except one of the two chromosome 4 loci and allowed distinction of each of the isolates from each other and from JH, except that the two isolates from the same household were identical to each other. In several cases, loci from different chromosomes yielded different phylogenetic trees, suggesting different histories for loci from different chromosomes. This observation could most readily be interpreted as being the result of recombination among different A2 genotype isolates. Even within the 9.5 kb chromosome 5 and 6 kb chromosome 3 loci, there were discrete regions with different phylogenetic histories. Algorithmic analysis using the HKY85 DNA substitution and GENECONV programs, yielded neighbor-joining trees with significant statistical support for meiotic recombination. These results are important not only to our understanding of the epidemiology of giardiasis, but may also affect views regarding eukaryotic evolution.

Introduction.

Giardia lamblia (syn. *Giardia intestinalis, Giardia duodenalis*) is an enteric protozoan parasite with two nuclei, which may be one of the earliest branching eukaryotes. Some of the cardinal eukaryotic organelles are absent from *G. lamblia* trophozoites, such as mitochondria, Golgi, and peroxisomes. Therefore, at one time *Giardia* spp. were thought to have diverged from the eukaryotic tree before the acquisition of these organelles. However, the subsequent identification of at least rudimentary forms of some of these organelles has led to the abandonment of this proposal. An organelle resembling Golgi was identified in encysting trophozoites, which is involved in protein trafficking (Reiner *et al.*, 1989). More recently, double membraned organelles thought to be mitochondrial remnants have been identified (Tovar *et al.*, 2003). Therefore, it remains possible that *Giardia* spp. are among the earliest branching eukaryotes, but it is unlikely that they predate any of the major eukaryotic features.

Giardia spp. have also been considered to be primitively asexual. Sexual reproduction has never been documented in *Giardia* spp. and population genetic studies have suggested clonal replication (Tibayrenc, 1993; Tibayrenc *et al.*, 1990). Subsequent molecular epidemiologic studies have generally assumed clonal rather than sexual reproduction of *G. lamblia.*

However, there are several questions that are difficult to answer if *G. lamblia* reproduces entirely asexually. First of all, it is difficult to explain how *G. lamblia* trophozoites are able to homogenize their two nuclei without some form of recombination. Trophozoites possesss five distinct chromosomes (Adam *et al.*, 1988)

with an estimated average ploidy of four during the G1 phase of DNA replication (Bernander *et al.*, 2001) and each nucleus contains a full complement of the genome (Yu *et al.*, 2002). Trophozoite replication maintains the left:right asymmetry of the nuclei (Yu *et al.*, 2002). Therefore, the nuclei would be expected to diverge from each other in the absence of some type of recombination, as has occurred with the bdelloid rotifers that have replicated asexually for millions of years (Welch & Meselson, 2000). Despite these expectations, the extremely low allelic sequence heterozygosity (ASH) in the genome isolate, WB, indicates that the DNA from each of the nuclei is nearly identical. Secondly, the extremely low level of ASH in the WB isolate is difficult to explain even for the two alleles within the same nucleus. It is also difficult to explain in the absence of sexual reproduction since mitotic crossing over is generally much less efficient at homogenizing double-stranded DNA than is meiotic replication.

In view of these observations, it is notable that a recent evaluation of the *G. lamblia* genome demonstrated that it has all of the genes that are necessary for meiotic recombination (Ramesh *et al.*, 2005). The complete significance of this observation is not yet clear since many of the meiosis-specific genes could potentially have other functions, such as other aspects of DNA repair. If sex occurs rarely, it may be very difficult to identify in the absence of a good repertoire of selectable markers. However, these data do suggest a potential role for population genetic approaches in re-evaluating this question. The early population genetic studies were performed using zymodeme analysis, which depends on finding amino acid mutations in enzymes that result in a change in charge. The studies have also been done in the absence of chromosomal

location of the loci being evaluated, and frequently without information regarding the specific genotype of the isolate. This is important because if the different genotypes represent different species there may be no recombination between genotypes. Extensive sequence comparisons have the potential of greatly increasing the likelihood of detecting molecular differences in isolates that could provide evidence for sexual replication and recombination. All *G. lamblia* isolates obtained from humans have been from groups 1, 2, or 3 (Nash & Keister, 1985; Nash *et al.*, 1985). These 3 groups have been designated as Genotypes A1, A2, and B, to reflect the relative similarity of groups 1 and 2 and the greater difference of group 3. The genotype A1 (eg. genome isolate WB) isolates are so homogeneous that it is extremely difficult to find any sequence differences among these isolates. On the other hand, the genotype B isolates are so different from WB that it is more difficult to use the WB genome as a "template" for obtaining sequence from comparable regions of the genome. Therefore, we have used a set of genotype A2 field isolates from an endemic region near Lima, Peru to evaluate the possibility of population genetic evidence for meiotic or other recombination.

Materials and Methods.

Sequencing Strategy:

Sequence identification. The JH isolate is a genotype A2 isolate that has been cultured axenically and was used as the A2 reference isolate for this study (Nash 1985). The region adjacent to the *triose phosphate isomerase* (*tpi*) gene on chromosome 5 (Genbank #L02120) was obtained from the WB (Genotype A1) genome isolate (www.GiardiaDB.org). A similar strategy was used for chromosome 3 on a region adjacent to γ-giardin gene (Genbank #X55287). The *glutamate dehydrogenase* (*gdh*) gene (M84604) and *β-giardin* (X85958) provided shorter loci from chromosome 4 (Table 1).

PCR amplification. Primers were designed to amplify 500 bp regions of genomic DNA and included at least 50 bp of overlapping DNA in order to identify any snps located within the chosen primer sequences. Primer pairs specific to chromosome loci (Table 1) were used for amplification (1μM concentrations). Concentrations of other PCR master mix ingredients were as follows: 1.5mM magnesium chloride, 1μM dNTPs, 0.5% non-acetylated bovine serum albumin (Sigma) and 10% dimethyl sulfoxide (DMSO, Sigma). Thermocycler conditions were as follows: 2 minute initial denaturing (94ºC), 40 cycles of 30 seconds denaturing (94ºC), 30 seconds annealing (51ºC), 1 minute extension (72ºC), followed by 10 minutes of final extension (72ºC). PCR products were purified using the microcentrifuge based Gel Extraction protocol of the QIAquick PCR purification kit

(QIAgen, Valencia, CA). Sequencing was performed on purified PCR products using nested amplification primers with the addition of DMSO.

Algorithmic analysis.

Exploratory tree analysis was used to determine if there was evidence of conflicting phylogenetic signal (Worobey & Holmes, 2001). Neighbor joining trees were reconstructed on 600-nucleotide (nt) windows of the original alignment shifted in 300-nt increments, as well as for the full-length data set, with distances estimated using the HKY85 (Hasegawa *et al.*, 1985) model of DNA substitution. Conflicting phylogenetic signal contained within the sequences of putative signals was confirmed by bootstrap phylogenetic trees. To complement the phylogenetic approach, Sawyer's (Sawyer, 1989) runs test, in the package GENECONV (S. Sawyer, Department of Mathematics, Washington University, St. Louis, Mo.) searches for unusually long fragments within an alignment over which a pair of sequences are identical or nearly identical, then assesses the significance of the hypothesis that the similar fragments arose by recombination by using randomly permuted data sets derived by the real alignment.

Table 1. Sequencing primers for three loci. Primers underlined denote those that were applied to field isolates for molecular typing purposes on a larger scale (manuscript in preparation).

Chromosome locus	**Primer 1**	**Primer 2**
adjacent to *γ-giardin* on chromosome 3	3c-1F TGGAGGCGGTCAAGATACTC 3c-1.5F CGGTCAAGATACTCTACGATCG 3c-2-F GGATTCAAGACAAACTCGCG 3c2-5F GCCTTTCTGAAGTCACTCATGCACACG 3c-3-F GTGCTATGGCATGGAGTGGG 3c-4-F GTCGATGAAGATGATGGTCG 3c-5-F CCAAGAGGGCATTGATACGC 3c-6-F CGATACAAGTGGATCGGTTGC 3c-7-F GCCTTGGCAGGCTTTGATGACG 3c-8-F GCGATTGATTAATGACCACCTGACG 3c8-5F CGTAAGCAGCGTGCAATACGACAGCG 3c-9-F CCTTCGTCGTCATGATCGCGTCG 3c9-4F GTGCTCATTGTTGGATATGGACG 3c-10-F CCTCAAGTCGGTAGACTCCAGCTCG 3c10-5F CCTCTGCTCCATCGAGAAGACCTCG	3c-1R GAGGTCATATGGAGTATCTG 3c-1.5R CCTCGACGATTATGCTCCACGACG 3c-2-R GACGCATCCGCGTGTGCATG 3c2-5R GCCAACCAACCGACCATCATCTTTATCG 3c-3-R GGCATGCGCTAATGAGACG 3c-4-R GCCATCCATAGGATGCCTG 3c-5-R CCTCAAATGAGCCAAACAGC 3c-6-R CAAGGTACTTACCCTTCTGC 3c-7-R GCACATACTCCTCGGGTGGAATCG 3c-8-R GATCAAGTCCTTCGCAGAGAGCG 3c8-5R CCAATCAGCTCCCCAAGAGTTGCG 3c-9-R CCAGGACCACATGGTGAACG 3c9-4R GACATTCTGTCCACAAAGAACATCCG 3c-10-R CCTGTTTGTGAATGACGATCCG 3c10-5R CCTGTGTTTGTGAATAAGAAATCCG
***β-giardin* and *gdh* on chromosome 4**	4c-1F GCTTCCTTAGTCAGCTCAGCACG 4c-2F CCTGCAGATGTACCTGCACGACC 4cg-1F GCGATCCTTAGCAATGTAAACG 4cg-2F GCTCATTCCTGCCATCTTCATCTCCG	4c-1R GCCTGGCTTTGGAGGAATGTCG 4c-2R CCAGAGTCTAGGATAAGGTGCC 4cg-1R GCCAACCACTTTCCCAGTCG 4cg-2R GCTCGAAACCGAGGAACTTGAGAATCG
adjacent to *tpi* on chromosome 5	5c-1F GAAGGGGCGACGAGCAGGCATG 5c-2F CCCATCTTCTTCAGCTCTGCATTA 5c-2.5F CCAGAGAGACATGCATATCAATC 5c-3F GCGTATCCAGAGCTATCTTACAAG 5c-3.5F GGCATCTATAAACAACGGCACGG 5c-4F GGCCCCCATTGTCCCTAAGC 5c-4.5F GCCAGTAAGAAAGATGTTCGCAA 5c-5F GGGTTGACGTTGTGCAAGAACG 5c-5.5F GCACGTGCTATACACAAGTG 5c-6F CCGAGGCCAAATATCGGGAGCC 5c-6.5F CCATGTCGGTGGCGGAAAGTATC 5c-7F CCCTTCCACATCGTTTACTACC 5c-7.5F GCATGATGGTTGCATTGAGATCG 5c-8F CGTGAGATGAGTATCCTCCGC 5c-8.5F CCTCAGACGGCACAAGCAAAAGG 5c-9F CCCTTGAAACTATTCGACCC 5c-9.5F GGAATGTTGAGTCCTTTACGG 5c-10F CCAGCCCTCAGACGAATACC 5c-10.5F TCCTGCTCTTCCAAACTGTATATG 5c-11F CGACAGAGTCCGTGCTGCTCACC 5c-12F GGCGAGTGCAGTCCTGAGTGG 5c-12NF CAGTTTGGAAGAGCAGGACTCG	5c-1R GGACTGGCAATTGCAGATAAGG 5c-2R GCGTAGTACTTTCCTGTGTCAATG 5c-2.5R TCGCAAGATACTACACTCG 5c-3R CGATCTCGTCCTCTTCAAAGTTAG 5c-3.5R AGGGAGCAAAAGGAGTAAGG 5c-4R GCAGCCATTAAAGGATCTAG 5c-4.5R AGAATTGATCAGCTCTGCTGCCT 5c-5R CCTTCGCTGCCAGGAGGAATGC 5c-5.5R GCCAATGATGCTGATCTTAAGCG 5c-6R CACCCTCTACTAATCCTGTTCC 5c-6.5R GGCTAAGAACAAGTCTCACCGAG 5c-7R CCTGATAGACACATATCCCATGGCC 5c-7.5R CGTTTATGGAATGTCTCGTTGCC 5c-8R GGGAAACAATCTTACAGGGTGG 5c-8.5R CTGAGCCAGCTCATCTTTGC 5c-9R GGTGTTCTTAGCTGCTCTATGC 5c-9.5R CGAAGTTCTTGCATAGACGATGC 5c-10R GCGCACACATACACCCGGGACCAGG 5c-10.5R CAGGGTCAGATAGACAACCACG 5c-11R GCACATTGTGAGCTTGCCGG 5c-12R CCTGGCTTGTTAACTACATCC 5c-12NR GTCATCTTTCTATGCCTTCTTCG

Results.

JH sequence from four loci. Bidirectional sequence data were obtained for a 9.5 kb region at the end of the *tpi* gene (chromosome 5), a 6.0 kb region adjacent to the *γ-giardin* gene (chromosome 3), the *gdh* coding region (chromosome 4), and the *β-giardin* coding region (chromosome 4). Five different samples from different humans in four A2-positive families were chosen for comparison to the well-characterized JH isolate. The field isolates consisted of 55, 246, and 335, which represented individuals from three different households, and 303 and 305, which represented siblings.

Distribution of snps in the four loci. Two snp maps were created, one for the chromosome 5 locus (Figures 2) and one for the chromosome 3 locus (Figure 3). WB and JH shared 98% similarity in the chromosome 5 region, 99% sequence in the chromosome 3 region, and 98% similarity in β-giardin coding region on chromosome 4. Based on this initial analysis, four distinct A2 molecular types were defined.

The *gdh* and *β-giardin* coding regions, widely separated on chromosome 4, were sequenced for the A2 reference isolate and the five clinical isolates. The *gdh* coding region did not yield any snps for any of the samples. However, the *β-giardin* sequences for the samples did yield discriminatory snps, which can be observed in the phylogenetic tree (Figure 1C). The tree generated by the *β-giardin* sequences showed different groupings from those found in chromosome 5 or chromosome 3. In fact, all three phylogenetic trees (Figure 1) yielded different groupings, which led to questions about the possibility of meiotic or other recombination among A2 genotypes in *G. lamblia.*

Open Reading Frames (ORFs). Inspection of the 9.5 kb region on chromosome 5 included ORFs of a hypothetical protein in *G. lamblia* (43-1590, reverse), cAMP-dependent protein kinase A catalytic subunit (1648-2727, forward), hypothetical protein (2780-5008, reverse), leucine-rich protein-2 and *G. lamblia* virus receptor protein (5011-7374, reverse), inositol hexakisphosphate kinase (7393-8322, forward), and hypothetical protein in *G. lamblia* (8319-9563, reverse). A tblastn (NCBI website) search of possible ORFs detected on the SeqBuilder program of Lasergene 6 package (DNAStar) demonstrated that most of the sequenced region included the aforementioned ORFs. The most heterogeneous area between A2 isolates was a 400 bp region at the distal end of the 9.5 kb that was located in the hypothetical protein ORF 1197 nucleotides long.

Inspection of the 6.0 kbp region on chromosome 3 yielded five ORFs, 26S proteasome ATPase subunit S4 (1044-2387, reverse), hypothetical protein in *G. lamblia* (2516-3658, reverse), partial CP6 gene for cathepsin L-like protease (3700-4989, forward), hypothetical protein in *G. lamblia* (4891-5826, reverse), and an unidentified ORF (5150-5797, forward). The most heterogeneous region was a 700 bp region at the frontal end of the 6.0 kb.

Evidence for different phylogenetic histories for the three loci. Interestingly, a visual inspection of the two snp tables demonstrates substantially different patterns for the two regions. For example, JH, 55, and 335 are nearly identical in the chromosome 5 region, but in the chromosome 3 region, all three are substantially different while 55 and 246 are nearly identical. The *β-giardin* and *gdh* coding regions are at widely separated loci on

chromosome 4. There were seven snps in the *β-giardin* coding region, but none in the *gdh* coding region. These data are displayed in phylogenetic trees (Figure 1). An unexpected finding was that the samples did not group the same between chromosomes 5, 3, and 4.

The apparently different inheritance pattern for loci on different chromosomes suggest the possibility of sexual or other recombination, since clonal propagation should result in similar inheritance for loci on different chromosomes. These data are especially interesting in light of the recent demonstration that the *G. lamblia* genome includes the full spectrum of genes required for meiosis (Ramesh *et al.*, 2005).

Meiotic recombination. A visual inspection of the snp alignments (Figure 2, 3) suggests the possibility of sites for meiotic crossing over even within the loci from individual chromosomes. In order to examine this possibility quantitatively, we performed an algorithmic analysis of the 6.0 kbp chromosome 3 region and 9.5 kb chromosome 5 region using the GENECONV (Sawyer, 1989).

GENECONV performs a series of comparisons between all pairs of sequences in an alignment and asks whether certain fragments are unusually alike. For example, if there is a stretch of 2 kb where two sequences are identical, despite being highly divergent across the remainder of their sequences, that 2 kb fragment might be detected by GENECONV as a putative mosaic region. If, after statistically correcting for multiple comparisons, that fragment still appears to be unexpectedly similar, it will be flagged as a "globally significant" fragment by GENECONV. A simple follow-up analysis with

phylogenetic trees inferred from different regions detected by GENECONV can then confirm whether certain sequences contain regions with conflicting evolutionary histories. In chromosome 3, GENECONV detected several globally significant fragments. In the first two, 1-2717 and 2718-5979, JH is the recombinant sequence. In the next globally significant fragments, with 4402-4836 and without 4402-4836, sample 335 is the recombinant sequence. For chromosome 5, GENECONV detected several globally significant fragments and suggested that the 9.5 kb region contained six subregions of conflicting history (data not shown). These different regions supported three different topologies. In chromosome 3, JH and 335 demonstrated clear evidence of recombination (Figure 4, A,B and C,D, respectively). Both JH and 335 were in different phylogenetic positions in different parts of the alignment, and are highlighted with boxes in Figure 4 (A-D).. These conflicting phylogenetic positions were supported by high bootstrap percentages (>99%) in all cases.

For chromosome 5, samples 246 and 303 were the recombinant sequences (Figures 4 E-G), with high bootstrap percentages.

Discussion.

The pattern of genetic heterogeneity among *G. lamblia* field isolates can provide useful for the elucidation of critical issues in the molecular epidemiology of this organism. The complexity of the molecular epidemiology of this organism is not limited to its gross taxonomy or species specificity, but extends to the very biology of its means of perpetuating itself. A central question is whether reproduction occurs sexually or asexually. Tibayrenc *et al*. established an initial precedent for the argument for asexual recombination in *G. lamblia* in their landmark publication (Tibayrenc *et al.*, 1990). Clonal replication predicts that DNA sequences from different loci will yield similar inheritance patterns even if the loci are from different chromosomes. It may be necessary to reconsider our approach to understanding this parasite based on the discovery of meiotic genes in a study by Ramesh *et al* (Ramesh *et al.*, 2005). The data in the current study demonstrates different groupings of the same samples on different chromosomes and supports that meiotic or other recombination is occurring in A2 molecular types of *G. lamblia*. Indeed, if meiotic recombination is occurring, it may contribute to our further understanding of the molecular epidemiology of *G. lamblia.*

The current study is not alone in presenting seemingly conflicting genetic information through subtyping. Interestingly, in reviewing some recent literature, there have been two studies in which genotyping at new loci such as the *elongation factor-alpha* gene produce "poor genetic resolution" (Traub *et al.*, 2004). A study on wastewater in Norway also mentions difficulties with genotype groupings where snp

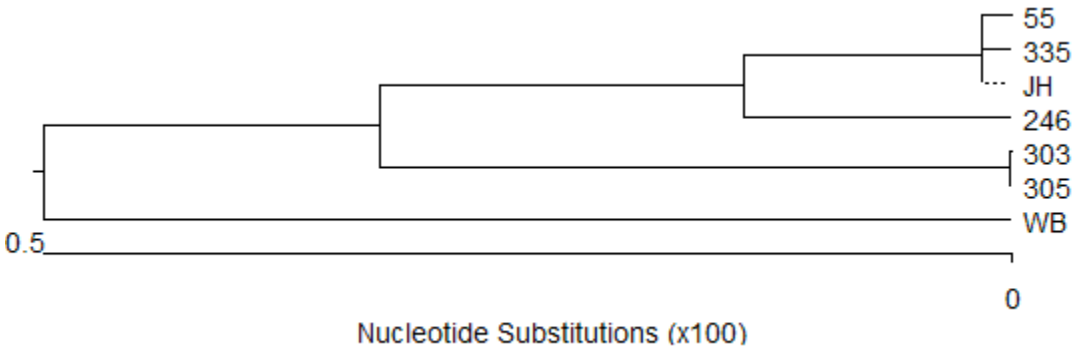
patterns with *gdh* match one particular molecular type but another molecular type with *β-giardin* (Robertson, 2006). It is possible that extensive sequencing of samples that present with conflicting results in a manner similar to the current study may yield more population evidence supporting meiotic or other recombination occurring in *G. lamblia.*

What would be the benefit of sex in *G. lamblia?* Bacterial sex in *Escherichia coli* has been correlated with higher rates of evolution in virulence and ability to escape the host immune response (Wirth *et al.*, 2006). In rapidly growing phagotrophic protozoa and starving dormant cysts, sex acts to balance conflicting selective forces (Cavalier-Smith, 2002). In *G. lamblia,* sex would explain not only how the microorganism homogenizes its two nuclei, but also explain the extremely low levels of ASH.

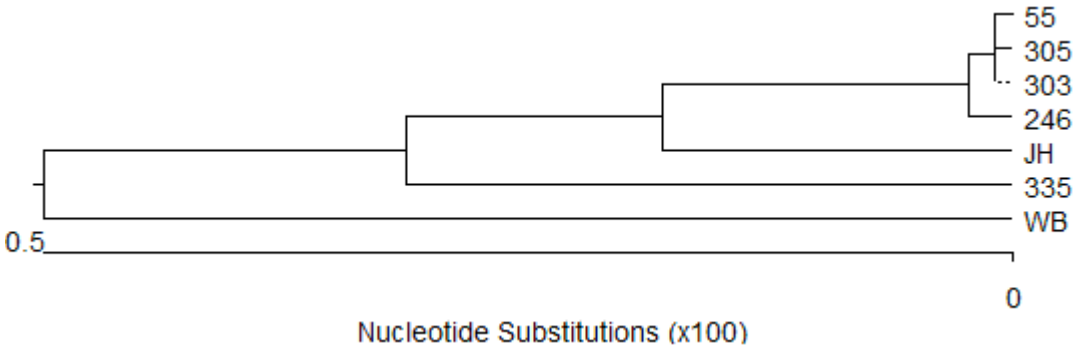
How *G. lamblia* could be undergoing sex, if indeed it is, has been pondered upon—is it infrequent, furtive, or cryptic (Birky, 2005)? Perhaps *G. lamblia* employs an alternative to meiosis, as in found in red algae and microcoporidia (Haig, 1993), or a precursor to meiosis (Birky, 2005).

In the event that *G. lamblia* is found to be undergoing sex in some fashion, its status as an ancient asexual eukaryote will be overturned. If that is the case, it may be found to be an ancient sexual microorganism, with relevance in its own right.

A.



B.



C.

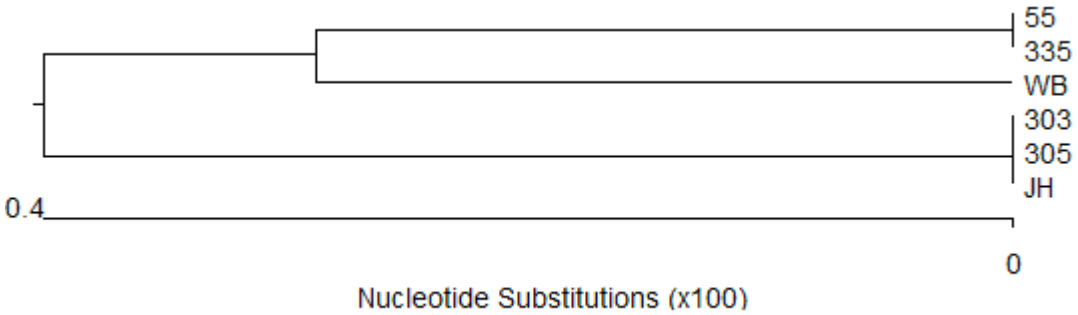


Figure 1. Clustal W constructed phylogenetic trees of sequences from chromosomes 3 and 5. A. Chromosome 5 9.6 kb region. B. Chromosome 3 6 kb region. C. Chromosome 4 1.2 kb region.

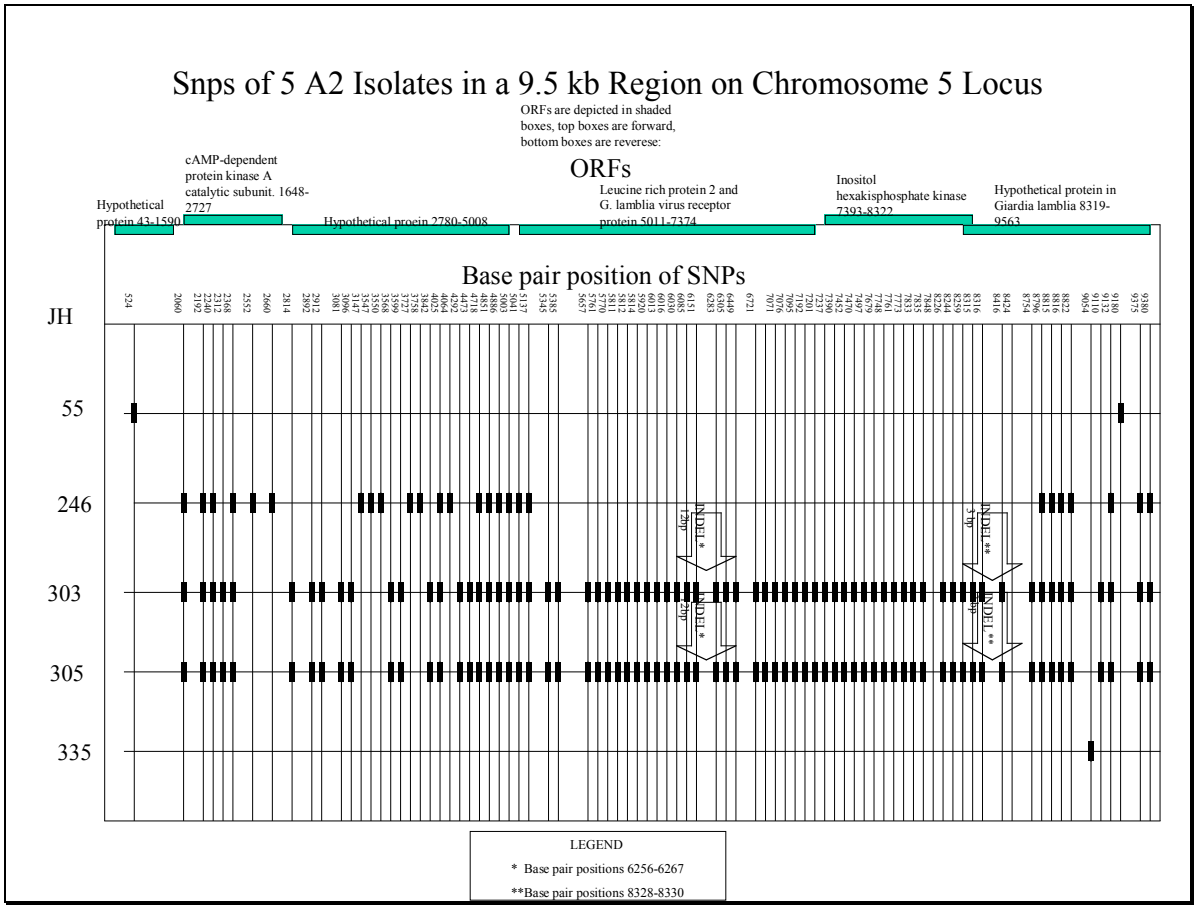


Figure 2. Chromosome 5 ORFs and snps 9.5 kb

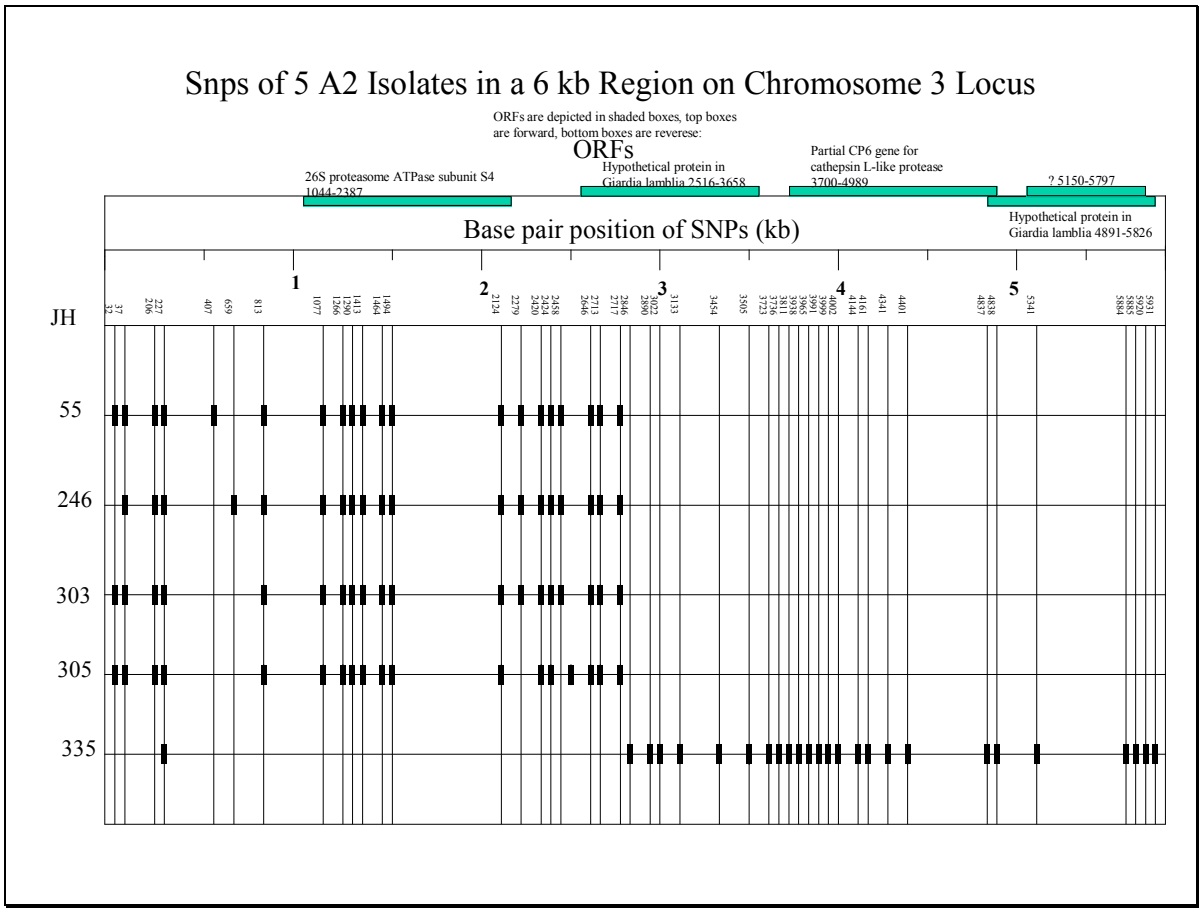
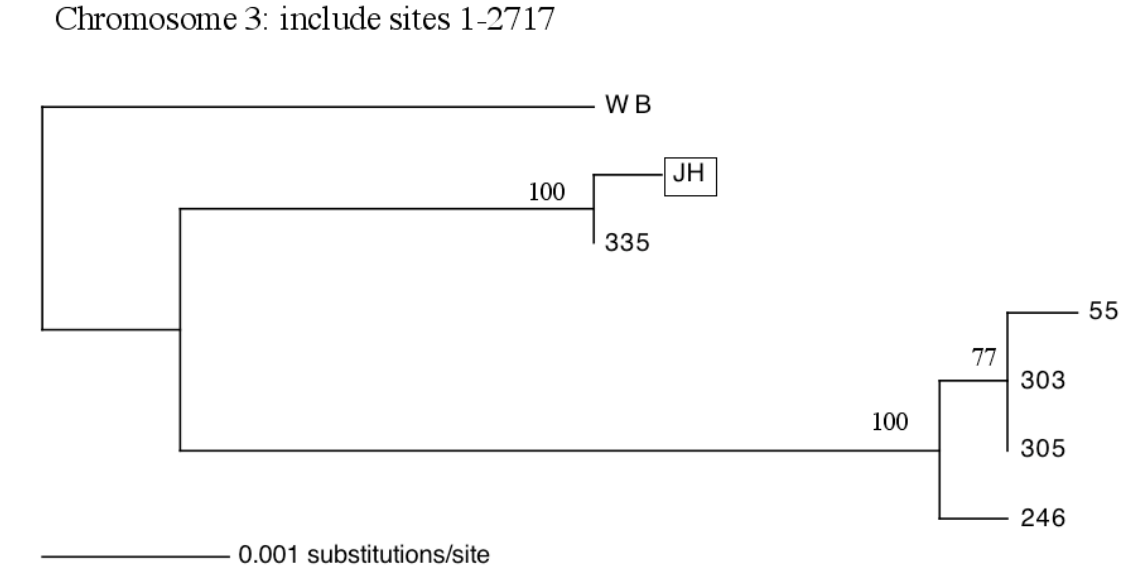
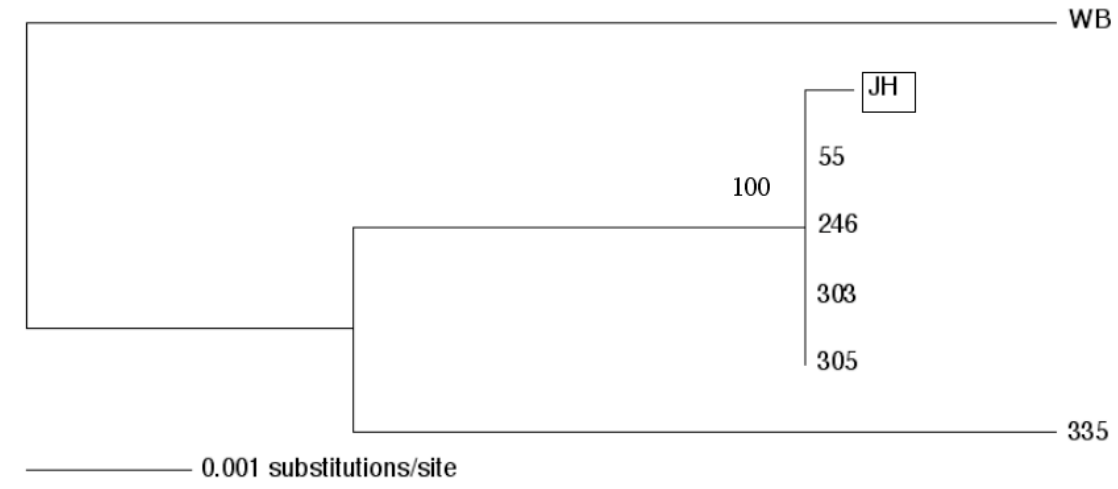


Figure 3. Chromosome 3 ORFs and snps 6 kb

A

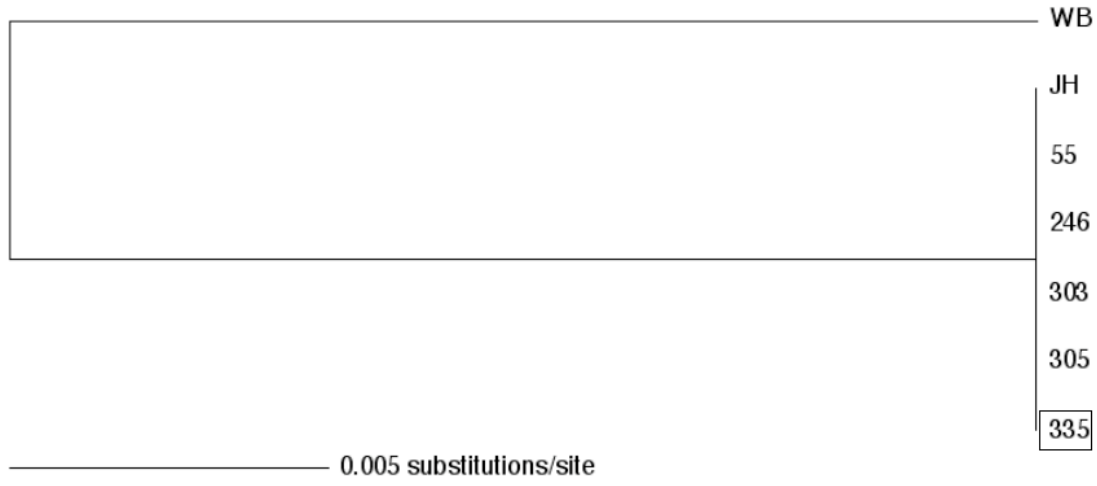

Chromosome 3: include sites 1-2717
W B
JH
100
335
55
77
303
100
305
246
0.001 substitutions/site

B


Chromosome 3: include sites 2718-5979
WB
JH
55
100
246
303
305
335
0.001 substitutions/site

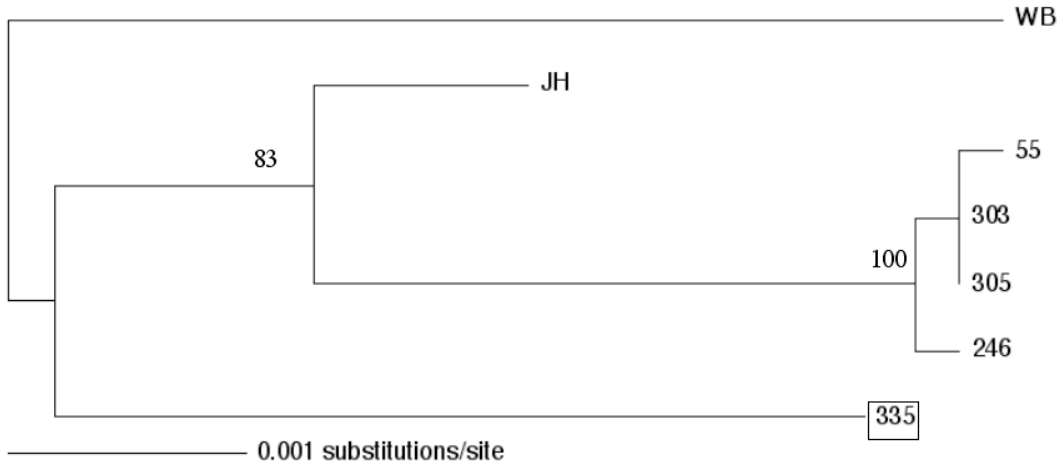
C

Chromosome 3: include sites 4402-4836



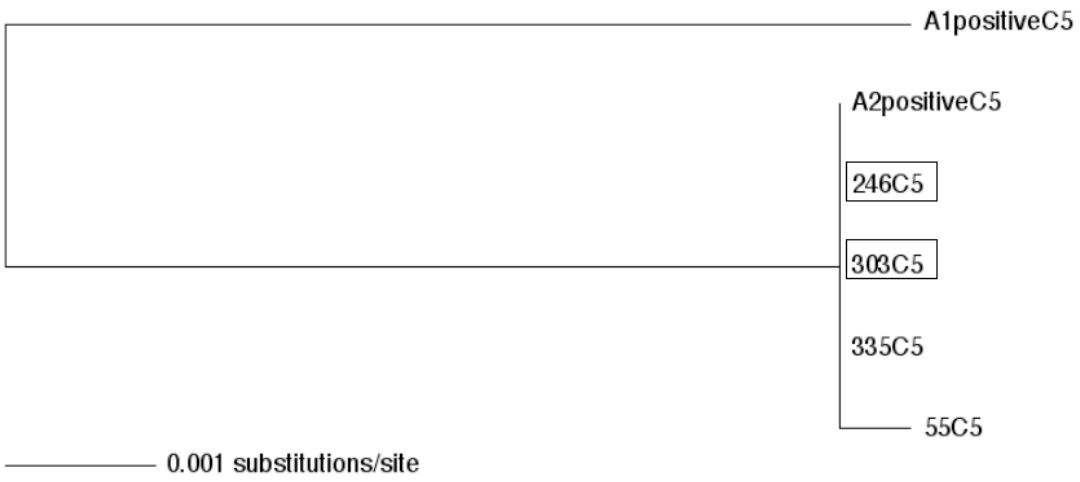
D

Chromosome 3: exclude sites 4402-4836



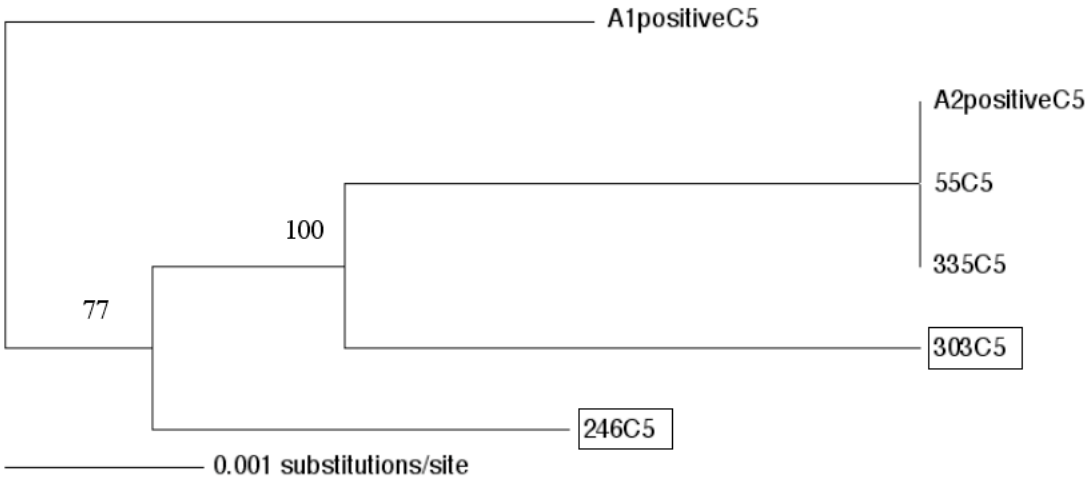
E

Chromosome 5: include sites 1-2057



F

Chromosome 5: include sites 2058-5137



G

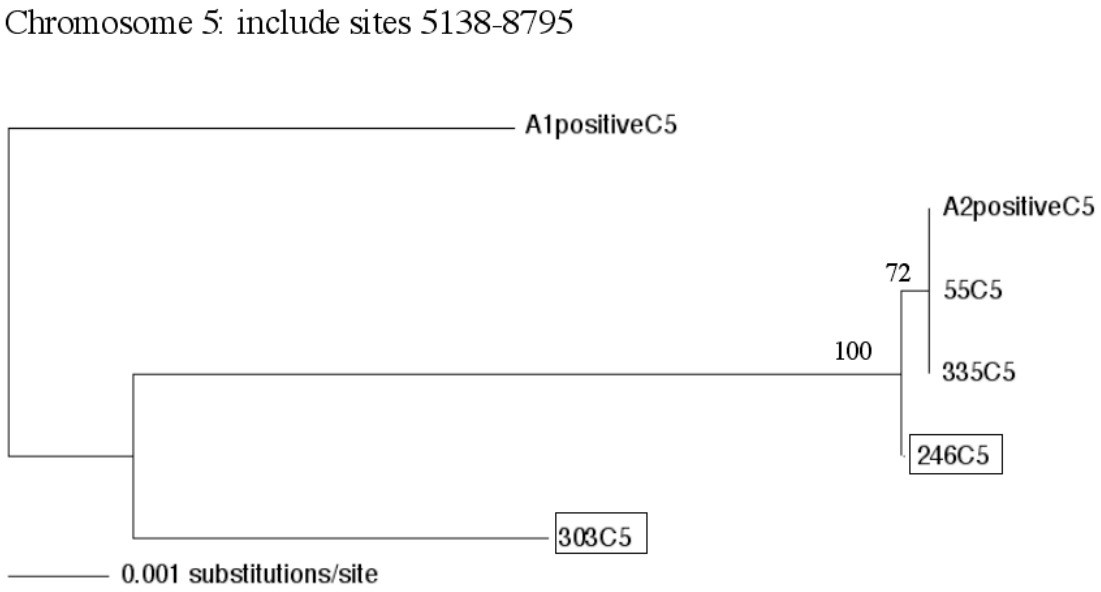


Figure 4. A-G. Evidence for sexual recombination in chromosomes 3 and 5. Boxes highlight recombinant sequences.

Acknowledgments. The author would like to thank Dr. Robert Gilman for his sponsorship of the sample collection project in Peru. The author would like to acknowledge Drs. B. Helen Jost, Donna Wolk, and Lynn Joens for helpful commentary. Lilia Cabrera was critical in sample collection at the study site. This study would not be possible without the assistance of P.R.I.S.M.A. and funding from National Institutes of Health Minority International Research Training grant TW00036.

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