12-2010

Study of Population Diversity of Toxoplasma gondii

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To the Graduate Council:

I am submitting herewith a thesis written by Debashree Majumdar entitled "Study of Population Diversity of Toxoplasma gondii." I have examined the final electronic copy of this thesis for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in Microbiology.

Chunlei Su, Major Professor

We have read this thesis and recommend its acceptance:

Benjamin Fitzpatrick, Todd Reynolds

Accepted for the Council:
Carolyn R. Hodges

Vice Provost and Dean of the Graduate School

(Original signatures are on file with official student records.)

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Study of Population Diversity of *Toxoplasma gondii*

A Thesis Presented

for the

Master of Science Degree

The University of Tennessee, Knoxville

Debashree Majumdar

December 2010

Dedication

I would like to dedicate this work to the memory of my dad, Dr. Buddhadeb Majumdar. You did not get to see me through this, but you were my inspiration every single day and I hope I have been able to make you proud. You never stopped looking out for me and I would never stop looking up to you.

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First and foremost I would like to thank my mentor, Dr. Chunlei Su for his constant guidance, constructive criticisms and supervision over the course of my graduate program.

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All my friends, especially Lydia, Sonia, Junwei and Marissa, I thank you for being there for me through good times and bad. Lydia thanks for holding my hand through the TA classes and for your good heart. Guys, I really appreciate our bonds of friendship and will forever cherish the memories we made here.

My parents have been the most important influence in my life and I could never thank them enough for all that they have done and all that they have been. Dad, not a day goes by when I don't remember how lucky I am to have had a loving father like you. You have trusted me, believed in me and supported me in all my pursuits including attending graduate school thousands of miles away from home. I want you to know that in my heart you are always with me and I will continue to follow the path shown by you. For all the times you lifted me up and helped me believe that anything is possible.... Thanks!

Ma, thanks for teaching me everything from the alphabets to the values of life, for encouraging me to reach for the stars and for raising me with a love of science. I hope you know how very much you are appreciated for all the sacrifices that you and dad made to help me succeed.

Finally, a big thank you to my significant other, Soumitra who has been my rock the entire time. His love, patience and support carried me through all the weekends I had to spend working in the lab, all the nights I had to sit up chasing deadlines and through the roughest times. I couldn't have done it without you.

ABSTRACT

Toxoplasma gondii, the causal agent of toxoplasmosis, is an important water and food borne protozoan parasite. *T. gondii* was previously shown to have a distinct clonal population structure composed of Type I, II and III lineages in North America and Europe. But more recent studies demonstrated high diversity in South America. In the present project we have conducted an intensive study of the population diversity of *T. gondii* and surveyed the extent of genetic variation among natural *T. gondii* isolates on a global scale in order to better understand the population dynamics and pathogenesis of this parasite. To this end, 948 *T. gondii* isolates have been collected from a broad range of animal hosts and different sites worldwide. Our initial multilocus PCR-RFLP genotyping analysis revealed high diversity (~140 distinct genotypes) with abundant unique genotypes in South America and a strong clonal population structure in North America, Europe, Asia and Africa. It also showed that the Type II is the most common lineage worldwide, followed by the type III strain. The Type I strain, though widely distributed, has been infrequently isolated. Several new clonal genotypes have been identified from South America. The newly identified 140 RFLP genotypes have been further analyzed by multilocus microsatellites and intron sequencing methods. The composite data set identified 11 different haplotypes, providing a framework for future study of molecular epidemiology and population genetics of *T. gondii*. Multilocus DNA sequencing of markers from each of the 14 chromosomes covering the entire genome has also been completed to help reveal more information about genome evolution and the origin of *T. gondii*. Taken together, this comprehensive epidemiological and population genetic study has revealed significant details on the diversity and extent of sexual recombination, which provides the basis for future studies to understand transmission patterns, population dynamics and origin of this successful apicomplexan parasite *Toxoplasma gondii*.

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Chapter 1 : Introduction

Introduction

Toxoplasma gondii, the causal agent of toxoplasmosis, is an important water and food borne protozoan parasite ubiquitous throughout the world.[8,59] It belongs to the phylum Apicomplexa along with other well-known members like- *Plasmodium, Sarcosystis, and Neospora*.[8] The Apicomplexa are an extremely large and diverse group of parasites comprising of ~5000 members and cause a variety of life-threatening diseases in humans and animals. [8] Pathogenesis is typically associated with the ability of these parasites to replicate and proliferate within host cells. However, pathogenicity can vary with the host and the stage ingested.[26-28,30] *Toxoplasma gondii* is a highly prevalent obligate intracellular parasite that has no host specificity and infects all warm-blooded vertebrates including mammals and birds.[8,67] It is the only known species in the genus *Toxoplasma* and is considered one of the most successful eukaryotic pathogen in the world in terms of the number of host species and percentage of animals infected worldwide.[8,64,102,106]

T. gondii research is a significant area of study because of its medical and veterinary importance.[8] In humans, *T. gondii* is transmitted by ingestion of tissue cyst, by ingestion of oocyst in contaminated vegetables and water or by congenital transmission.[8,67] *Toxoplasma* infection is a serious threat to immunocompromised individuals such as AIDS patients and organ transplant recipients. It can cause severe and life-threatening disease (e.g. encephalitis, retinitis, and myocarditis) in developing fetuses and in immune-compromised patients.[11,67] Up to one-third of the human population in the world is chronically infected and more than 60 million people in United States itself are believed to be infected.[8,106] (***Toxoplasmosis***: Fact Sheet - CDC DPD). *Toxoplasma* infection is also implicated in etiologies of neurodevelopmental and neurocognitive disorders like-schizophrenia.[107] Although current available drugs like-pyrimethamine and sulphadiazine, can control the proliferative form of the parasite and treat *Toxoplasma* infections, they are poorly tolerated, have severe side effects like allergic reactions and are ineffective against chronic *Toxoplasma* infections. In addition, resistance to some of these drugs has recently been noted. [5,6,11,19]

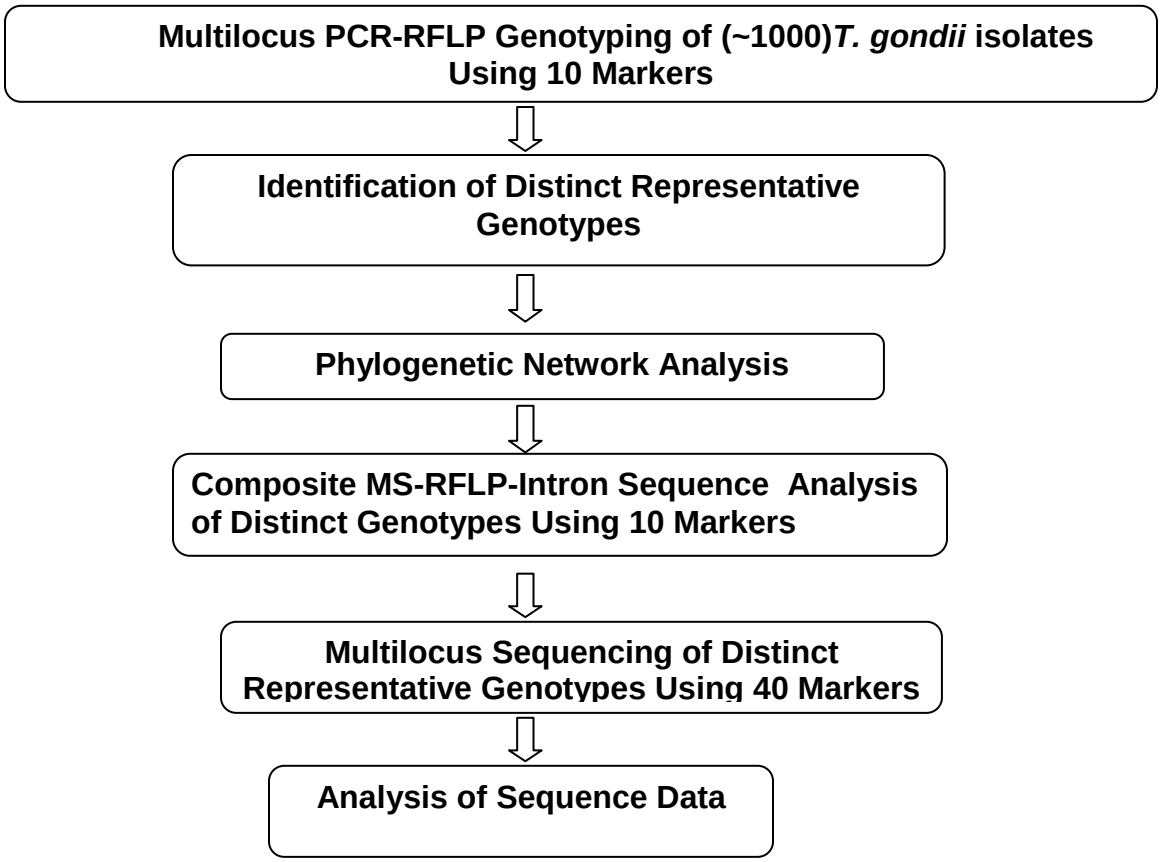
The immense success of *Toxoplasma gondii* is due to its spread in different environments through resistant oocysts shed by felids and by transmission between intermediate hosts through

carnivorous or omnivorous feeding.[8,60,67] It is also due to the ability of asexual forms to penetrate and grow in virtually all types of animal cells and its long-term survival as tissue cysts.[8,67] Such adaptation to different hosts suggests a large genetic diversity.

For a long time, *Toxoplasma gondii* was considered to be clonal with three genetic types (Types I, II, III),[4,20,67] but recent studies on *T. gondii* populations in different animal populations worldwide started to reveal the extensive diversity of the parasite.[31,80,91] In addition, there is extensive evidence that the clinical outcome of the infection varies with the *Toxoplasma* strain.[4,63,69] Hence, there is a real need for clarification of *T. gondii* population structure.

So in the present study, we analyze the population diversity of *Toxoplasma gondii* on a global scale in order to: 1) determine the extent of genetic variation among natural *T. gondii* isolates; 2) develop a DNA barcode system to accurately determine the genotype of clinical isolates in the future; 3) define the possible association of parasite genotype with specific host species; 4) investigate if the genotypes are related to disease manifestations in human toxoplasmosis; and 5) reveal global patterns of *T. gondii* transmission. Moreover, clear correlations between genotype of the infecting strain and its clinical course of infection will aid in developing more effective prevention, treatment and control strategies. *Toxoplasma gondii* can also serve as a model system for other disease causing Apicomplexan parasites including *Plasmodium* (the causative agent of malaria); *Eimeria*, (the cause of poultry coccidiosis); and *Cryptosporidium*, (an important opportunistic infection in AIDS patients).[8,111] Data obtained from this proposed study will help reveal the population diversity, intensity of sexual recombination and the dynamics and origin of *T. gondii* population. Taking all this into consideration, a set of experiments has been conceptualized for the present study and the complete experimental design is illustrated below in a step wise flow chart.

Experimental Design –



Chapter 2 : Background and Significance

Background and Significance

T. gondii ranges from 15-85% in its worldwide prevalence with the incidence of *Toxoplasma* infection in France being greater than 90% among adults.[8,67] *Toxoplasma* parasitizes and infects virtually any bird or mammal, even those who live exclusively in the sea, making it unique among other eukaryotic infectious agents.[8,11,41,67] (Fig 2.1)

2.1 Transmission

There are three principal routes of transmission of the parasite: ingestion of infective oocysts shed by the cat, carnivory (consumption of raw or undercooked meat) and congenital transmission.[8] Human infections are primarily caused by consuming uncooked meat containing viable tissue cysts or by ingesting food or water contaminated with oocysts shed by the cat (Fig.2.2). Although *T. gondii* can be transmitted in several ways, it has adapted to be transmitted most efficiently by the cats-intermediate hosts-cats route. The high transmission efficiency and prevalence of the parasite is largely attributed to the widespread presence of its definitive hosts (felines) across much of the earth from equatorial rain forests to tundra-like vegetations in high elevations and extreme latitudes.[13] Moreover, sero-epidemiological studies on isolated islands in the Pacific[110], Australia[86] and the United States[29] where very few cats are present, have shown a very low seroprevalence of *T. gondii*, confirming the important role of the cat in the natural transmission of *T. gondii.*

Another route of transmission of *T. gondii* infection in humans is via congenital infection and is often associated with severe pathology in the foetus. In humans, transmission of *T. gondii* from the mother to the foetus is considered to be most efficient during the last trimester of pregnancy but clinical congenital toxoplasmosis is more severe if transmission occurs during the first trimester.[18,23,44] Congenital *T. gondii* infection in a human child was initially described by Wolf, Cowen, and Paige[113] and later found to occur in many species of animals, particularly sheep, goats, and rodents. Congenital infections can be repeated in some strains of mice with infected mice producing congenitally infected offspring for at least 10 generations [9]. Epidemiological evidence indicates it is common in humans in localities where raw meat is routinely eaten.[22] It is very likely that the high degree of ubiquity and prevalence of *T. gondii* is generated by an interaction of all three transmission routes.

2.2 Symptoms

Primary infections in normal healthy adults are mostly asymptomatic but in some patients lymphadenopathy or ocular toxoplasmosis can occur.[15,112] In immunocompromised individuals, *T. gondii* can cause life-threatening encephalitis.[84] In pregnant women, symptoms range from congenital blindness, mental retardation to even death of the fetus.[71] Brain infection by *Toxoplasma* may cause hydrocephalus, intracerebral calcification and chorioretinitis in neonates, or more subtle changes in brain development, implicated in the multifactorial etiologies of epilepsy and schizoaffective disorders.[107] No human vaccine or drug against the brain cysts has been developed. The most devastating disease outcome is miscarriage or abortion which is particularly important in humans and domestic livestock.[89,111] The conventional diagnosis of *T. gondii* infection usually employs serological tests, bioassays in cats and/or mice, or a combination of the two approaches.[8] In the past two decades, the diagnosis of *T. gondii* infection by direct detection of parasite-specific DNA in biological samples using molecular methods has gained popularity.

2.3 Life-cycle

T. gondii has a complex life cycle that includes sexual and asexual replication.[8,67] (Fig.2.2) Sexual replication of parasite only occurs within enterocytes of the cat intestine which acts as the definitive hosts whereas asexual replication takes place in any warm-blooded vertebrate which serve as intermediate hosts. Oocysts are shed by cats into the environment and they find their way to the intermediate hosts via contaminated soil, food and water. After several days of replication as rapidly growing highly infective tachyzoites during acute infection,[67] the parasites switch to slowly growing bradyzoites, which are encysted to form tissue cysts and reside in host cells for the life of the host as chronic infection.[25] Cellular differentiation of *Toxoplasma gondii* from the tachyzoite to the bradyzoite tissue cyst stage is the underlying cause of chronic toxoplasmosis. These bradyzoites may reactivate and convert to tachyzoites if host immune response is compromised, such as in AIDS patients.[84] *T. gondii* is the only known apicomplexan that can transmit directly orally among intermediate hosts and cause infection without cycling through its definitive feline host and this provides it the potential for clonal spread among wide range of intermediate hosts. [1,97]

2.4 Population Structure

North America and Europe. Clonality is the predominant pattern in North America and Europe, composed of Types I, II and III lineages.[3,67,96,109] Identical multilocus genotypes, a high degree of linkage disequilibrium between markers and a relative absence of recombinants exhibited by the majority of isolates are strong indicators of clonality. The three predominant clonal lineages differ by only 1–2% at the nucleotide level.[62] However, a small number of isolates highly divergent from the three major lineages and referred to as 'unique, exotic or atypical genotypes' are also occasionally found.[67] The clonal population structure of *T. gondii* can be best explained by a genetic bottleneck followed by a rapid expansion that resulted in the emergence of three predominant lineages. This pattern might reflect an unusual combination of genes, shared by the three lineages, that is responsible for their successful expansion. Several adaptive traits, including evolution of oral transmission between intermediate hosts, [100] enhanced transmission by domestic cats or adaptations to domestic rodents [80] might explain this expansion. Alternatively, the pattern of clonality may simply reflect an unusual demographic process that dramatically restricted the gene pool.

South America and other geographical regions. Recent population genetic studies of *Toxoplasma gondii* from South America and other geographical regions have revealed high frequency of non type I, II and III genotypes.[4,79] Current reports from Brazil and parts of S. America based on RFLP markers, and similar studies using MS markers, showed that isolates of *T. gondii* are biologically and genetically different from those in North America and Europe.[79,80,91,101] For example, highly unusual genotypes were detected in individuals who contracted toxoplasmosis while in the jungles of French Guyana.[16,17,21] This conclusion was subsequently echoed by numerous other studies of isolates from various animal hosts in Central and South America.[91] Similar diverse *T. gondii* genotypes observed both in human and animal samples in South America, [58,73] suggest that the divergence of isolates in South America is not due to host specificity but rather due to geographical differences.

The present understanding of *T. gondii* population diversity imply that parasite populations still largely appear to be dominated by a few essentially clonal lineages and sexual recombination is relatively rare in the field, with successful clones dispersing through asexual transmission of recombinants with distinct biological phenotypes. But the ability to recognize

parasite diversity has increased in parallel with increasing resolution of genotyping technology, and generated the need for a detailed study to redefine the existing population structure. [79,80,101]

2.5 Genotyping Methods

A variety of different molecular diagnostic methods (MLEE, microsatellite, RAPD, Intron sequencing, PCR-RFLP) exist today for carrying out in-depth population diversity and epidemiological studies of the parasite. [2,20,65,69,77,96]These different genotyping methods have been used in different labs worldwide and they each have distinct advantages and disadvantages. Early studies of strain typing were based on multilocus enzyme electrophoresis (MLEE). Several polymorphic enzymes were used to characterize *T. gondii* isolates largely collected from France, grouping them into three major zymodemes Z1, Z2 and Z3. [20] Though MLEE is quite specific, it requires a large number of purified parasites to perform. Later typing methods focused on microsatellite (MS) markers,[3,2,10] which are short repeated segments of DNA that tend to occur in non-coding DNA. MS markers have high mutation rates and thus enhanced resolution. Randomly amplified polymorphic DNA (RAPD) was also used to characterize strains of *T. gondii* [65]; however, this technique is highly influenced by contaminating host DNA, which can be a significant source of variability. Of late, restriction fragment length polymorphism (RFLP) analysis of specific genetic loci has been widely used to characterize *T. gondii* isolates.[67,69,96,101] This genotyping method has proved to be simple, sensitive, reproducible and cost-effective, and has been applied to a variety of clinical samples from animals and humans.

2.6 Origin and Evolution

It is suggested that the North and South American strains of *T. gondii* were split and separated from each other for over a long period of time during which they accumulated characteristic SNPs by random mutation and drift.[77] By calculating the extent of geographical allelic diversity in *T. gondii*, it was estimated that this North vs South America split occurred approximately 10^6 years ago.[77] Establishment of the Panamanian land bridge approximately 10^6 yr ago has been suggested to be responsible for migration of *T. gondii* into South America with cats and its subsequent diversification once introduced into the South . [72,77,82]

On the other hand, within the last 10^4 years, the three predominant lineages are believed to have evolved from a common ancestor, expanding very rapidly worldwide to populate a variety of hosts.[100] This is around the same time as the domestication of

agricultural animals, as well as the adoption of companion animals such as domestic cats.[24] A number of sexually differentiated parasites of agricultural animals have also been found to exhibit similar low genetic diversity, reflecting genetic bottlenecks and anthropogenic expansions in their recent ancestry.[94]

Another signature feature of *T. gondii* genome related to origin and ancestry is the existence of mostly a single monomorphic version of chromosome 1a (Chr1a*), as revealed by comparative genomic sequencing.[75] Based on the rate of somatic mutations between isolates within the lineages, it was estimated that Chr1a* arose approximately 10^4 years ago, probably coincident with the origin of the lineages. [75] The odds of all three lineages acquiring this same exact Chr1a* by chance have been estimated to be at least 1:1000 [75], suggesting this pattern arose owing to a selective advantage.

2.7 Genotypes and Biology

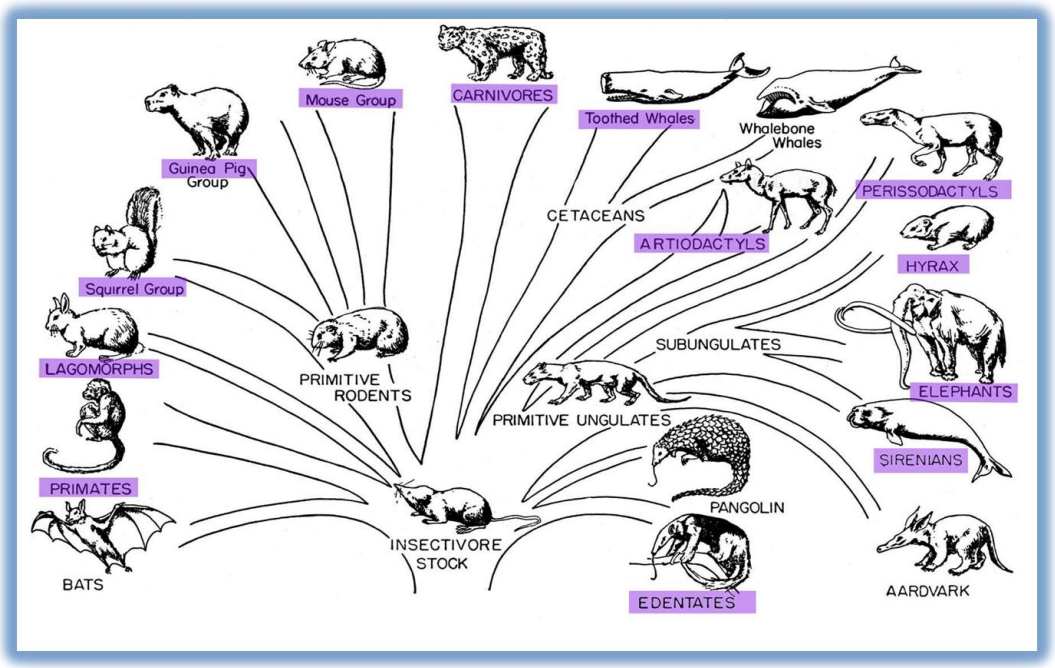
2.7.1 Association of parasite genotypes with biological hosts

The association of host species with *T. gondii* genotypes is of interest. An early survey indicated that both type II and type III strains are common in animals.[67] Several studies among agricultural animals, mostly from North America and Europe, have shown that the majority of isolates are type II, including pigs in the USA [45,83] and sheep from Britain.[89] But chickens in North America exhibit a higher prevalence of type III strains than type II [32]. In humans too, certain strains were more frequently associated with a particular type of toxoplasmosis in human patients.[61,63] Though sampling strategies in humans are notoriously difficult to conduct and are often based on clinical cases, type II strains are most commonly associated with human toxoplasmosis, both in congenital infections and in patients with AIDS.[66,67,69] The reasons for the differences between animal (types II and III) and human (largely type II) infections are unclear. At present, there are not enough data to statistically confirm association of host species with *T. gondii* genotypes.

2.7.2 Association of parasite genotypes with phenotypes

Biologically, the *T. gondii* clonal lineages differ in a number of phenotypes such as growth, transmission, virulence in laboratory mice and importantly, infectivity in humans.[7,85] Using forward genetic mapping studies to identify genes that determine natural differences in the

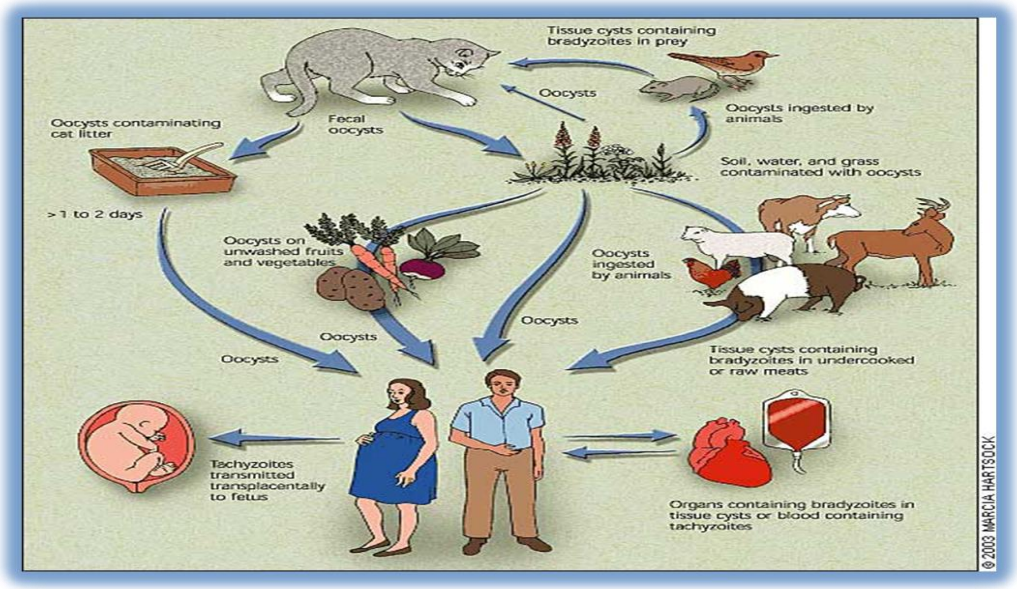
virulence of *T. gondii* in the mouse model, secretory protein kinases discharged from apical organelles, called rhoptries (ROPs) have been implicated as the key determinant of acute virulence by independent experiments.[78,95,105] Type I strains are acutely virulent and cause lethal infection in all strains of laboratory mice even at low inocula [lethal dose (LD_{100})= 1 viable tachyzoite], whereas types II and III strains are much less virulent [median lethal dose (LD_{50}) $\geq 10^5$ tachyzoites].[68,85,98] In humans too, like in mice, disease manifestations are highly variable, ranging from asymptomatic to severe, especially in cases of brain and eye infection.[12] Type II strains are most commonly recorded in human congenital and immunodeficient infections.[66,67,69] However, type I or type I-like atypical isolates are more likely to be involved in severe toxoplasmic retinochoroiditis in human patients[63] and the atypical isolates often cause severe acute, disseminated toxoplasmosis in immunodeficient patients. Interestingly, atypical *T. gondii* strains have been reported to cause acute disseminated toxoplasmosis even in immunocompetent individuals from S. America.[87,14] In the US too, from the more recent surveys, there is increasing reports about the presence of atypical isolates from food animals, particularly pigs and lambs, reflecting environmental contamination and potential source for human infection.[50] Such new variants might arise by somatic mutations, or result from sexual recombination between the major lineages. Deciphering these would require further sequence-based analysis of these new genotypes. Establishing the right connections among the type of strain and its disease causing potential in different geographical regions and in various disease scenarios will also substantially improve the management of human disease, matching an aggressive infection with an equally aggressive treatment.



(Romer and Parsons 1977)

Fig. 2.1: Known Hosts of *Toxoplasma gondii.*

It infects almost all warm-blooded vertebrates. Highlighted fields refer to the animal groups with reported *T. gondii* infection.



(http://www.aafp.org/afp/20030515/2131.html)

Fig. 2.2: *Toxoplasma gondii* Life Cycle.

Sexual replication takes place in cats and asexual replication occurs in intermediate hosts. Human infections are caused due to ingestion of oocysts, consumption of undercooked meat, transplacental transmission or infected organ/blood transfusion.

Chapter 3 : Multiplex Multilocus Nested PCR-RFLP Genotyping (Mn-PCR-RFLP) of *T. gondii* Isolates

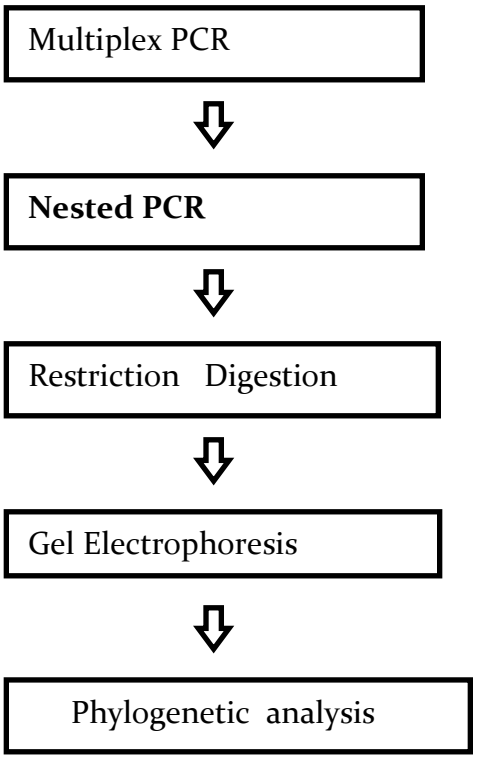
Multiplex Multilocus Nested PCR-RFLP Genotyping (Mn-PCR-RFLP) of *Toxoplasma gondii* Isolates using 10 markers

3.1 Introduction

Most *Toxoplasma gondii* isolates from patients and livestock in Europe and North-America, fall into one of three genetically distinct lineages: the Type I, II and III strains. But recent population genetics studies of *T. gondii* from animals and humans worldwide have revealed high frequency of non-type I, II and III genotypes. Previous population diversity studies had significant limitations in terms of sample size, breadth of collection sites and number of genetic loci examined, making it difficult to estimate the frequencies of different genotypes and to evaluate their association with disease presentation in humans. To overcome the existing limitations and reveal the diversity of *T. gondii* genotypes, it was necessary to take a systematic approach and analyze a large number of *T. gondii* isolates worldwide by multilocus genotyping and subsequent sequencing of the parasite DNA sequences. Thus the present study focuses on surveying the extent of genetic variation among natural *T. gondii isolates* on a global scale using multilocus PCR-RFLP method as the first step in order to reveal the true genetic diversity of *T. gondii*.

Multiplex multilocus nested PCR-RFLP (Mn-PCR-RFLP): PCR-based molecular methods are simple, sensitive, rapid, reproducible and cost-effective tools for the detection and genetic characterization of *Toxoplasma gondii* DNA in biological samples.[40,67] (Fig. 3.1) These methods have been applied to a variety of clinical samples from animals and humans in order to generate maximum information for epidemiological, population and phylogenetic studies of this key pathogen.[40,50,73,91,103,109] The advantage of this approach is that only a limited amount of individual sample is needed, which is particularly useful when only small amounts of DNA samples are available.[40,102] Application of this method to a broad range of samples will broaden our understanding of molecular epidemiology and population diversity of *T. gondii* in the near future.

Stepwise Flow Chart



3.2 Materials and Methods

Multilocus PCR-RFLP markers: A set of 10 unlinked PCR-RFLP markers including SAG1, SAG2, SAG3, BTUB, GRA6, c22-8, c29-2, L358, PK1 and Apico, each of which had the resolution to distinguish the three clonal lineages (type I, II and III) were used to characterize the isolates. [40,102]

***Toxoplasma gondii* strains:** A total of 948 *T. gondii* isolates were analyzed by restriction fragment length polymorphism (RFLP) method in collaboration with other labs in the world. These isolates were collected from a variety of animals and humans across all the major continents across the globe.[33,34,39,38,37,36,35,41,42,40,43,46,47,45,48,51,52,90-93,101,103,109]

PCR Controls: Strains RH (Type I), PTG (Type II) and CTG (Type III) were used as primary reference strains for genotyping. Additional positive control strains like- TgCgCa1 (COUG), MAS, HFF, TgCatBr5, TgCatBr64 and negative controls without DNA template were also included in each batch of experiment to monitor efficiency of PCR amplification and genotyping.[102] (Fig 3.2)

Mn-PCR-RFLP: In Mn-PCR-RFLP typing, all markers are preamplified by multiplex PCR using external primers in a single reaction, and the pre-amplified PCR products are then used as templates in the next step, to amplify each individual marker by nested PCR.[102] (Fig 3.1) The sensitivity of this method is estimated at 10 or more *T. gondii* genome equivalents, based on a previous study with 4 (SAG2, SAG3, BTUB and GRA6) of these 10 markers.[74] In contrast, the sensitivity of conventional PCR-RFLP is estimated at ~100 *T. gondii* genome equivalents. [102]

R.E Digestion: For each marker, the final PCR products were digested by specific restriction enzymes, and the strain-specific DNA banding patterns resulting due to the presence of sequence polymorphism within each marker locus were revealed in agarose gel electrophoresis. The distinctive RFLP patterns were compared to the reference type I, II and III strains to decide its genotype at that locus. The combined results of genotypes at all loci were used to determine the genotype of every individual isolate.(Fig 3.2) The primers and restriction enzymes used in this study for Mn-PCR-RFLP typing of *T. gondii* isolates are referenced from the recent publications. [40,102]

Phylogenetic Analysis: To reveal the phylogenetic relationship of all these parasite isolates, the complete dataset of these multilocus PCR-RFLP genotypes was analyzed by SplitsTree4 program and the phylogenetic tree was generated.[70]

Virulence studies: To determine if there is an association between multilocus genotypes and virulence phenotypes in mice, data of virulence in mice from the composite dataset was summarized from previously published reports. Mouse virulence of major genotypes was estimated based on mortality rates of three or more isolates belonging to the same genotype.

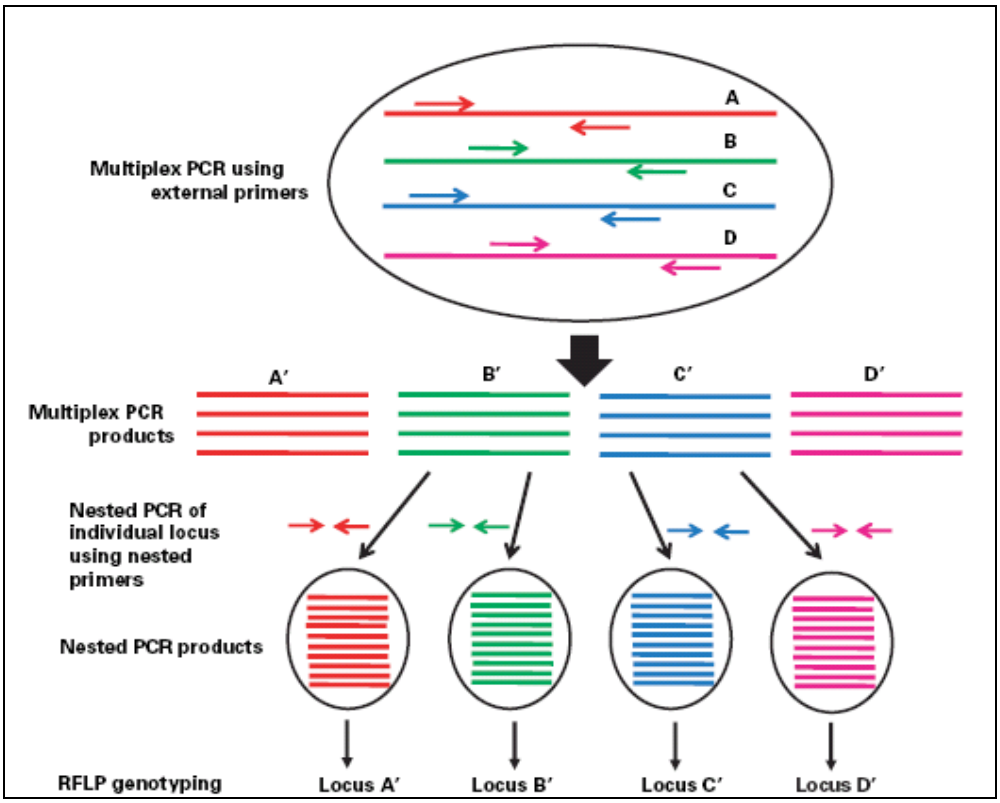


Fig. 3.1: Multiplex multilocus nested PCR-RFLP (Mn-PCR-RFLP) typing.
Horizontal lines represent DNA sequences and the horizontal arrows represent PCR primers.[102]

3.3 Results and Discussion

For our preliminary investigation, we conducted multilocus PCR-RFLP genotyping studies of *T. gondii* isolates from different animal and human hosts worldwide and compiled genotyping results from numerous recently published reports to summarize and estimate *T. gondii* genetic diversity. (Table 3.2) The genotyping results were published in a series of reports and following are the abridged versions of some of these recent research publications that I have been a part of and they showcase results of our PCR-RFLP genotyping of *T. gondii* isolates from different parts of the world. These reports are published under the same titles in different scientific journals and constitute a significant part of my research. The findings of these reports are summarized in Table 3.1 and the original published papers with the full details are attached in the appendix.

3.3.1 Published results-

I. Genetic diversity among sea otter isolates of *Toxoplasma gondii* [103]

N. Sundar , R.A. Cole, N.J. Thomas, **D. Majumdar**, J.P. Dubey , C. Su
Veterinary Parasitology 151 (2008) 125–132

Sea otters (*Enhydra lutris*) have also been reported to become infected with *Toxoplasma gondii* and at times succumb to clinical disease. Genotypes of 39 *T. gondii* isolates were determined from 37 sea otters in two geographically distant locations (25 from California and 12 from Washington). From these 39 isolates, Six distinct genotypes were identified using 10 PCR-RFLP genetic markers including SAG1, SAG2, SAG3, BTUB, GRA6, c22-8, c29-2, L358, PK1, and Apico, and by DNA sequencing of loci SAG1 and GRA6 in 13 isolates. The presence of six distinct genotypes from sea otters is an indication that the otters were infected with the parasites from different sources with possible genetic recombination. Of these 39 isolates, 13 (33%) were clonal Type II which can be further divided into two groups at the locus Apico. Two of the 39 isolates had Type II alleles at all loci except a Type I allele at locus L358. One isolate had Type II alleles at all loci except the Type I alleles at loci L358 and Apico. One isolate had Type III alleles at all loci except Type II alleles at SAG2 and Apico. Two sea otter isolates had a mixed infection. Twenty-one (54%) isolates had an unique allele at SAG1 locus. Further genotyping or DNA sequence analysis for 18 of these 21 isolates at loci SAG1 and GRA6 revealed that there were two different genotypes, including the previously identified Type X (four isolates) and a new genotype named Type A (14 isolates). The results from this study suggest that the *T.gondii* isolates from the sea otter are genetically diverse.

II. High prevalence and abundant atypical genotypes of *Toxoplasma gondii* isolated from lambs destined for human consumption in the USA [45]

J.P. Dubey, N. Sundar , D. Hill, G.V. Velmurugan, L.A. Bandini , O.C.H. Kwok, **D. Majumdar**, C. Su
International Journal for Parasitology 38 (2008) 999–1006

Lamb meat can be an important source of *T. gondii* infection for humans and there is a potential that more virulent parasite strains may circulate in animal reservoirs and subsequently transmit to humans. Little information is available on the presence of viable *Toxoplasma gondii*

circulating in tissues of lambs worldwide. The prevalence of *T. gondii* was determined in 383 lambs (<1 year old) from Maryland, Virginia and West Virginia, USA. In total, 53 isolates of *T. gondii* were obtained from 68 seropositive lambs. Genotyping of these 53 *T. gondii* isolates using 10 PCR–restriction fragment length polymorphism markers (SAG1, SAG2, SAG3, BTUB, GRA6, c22-8, c29-2, L358, PK1 and Apico) revealed 57 strains with 15 genotypes. Four lambs had infections with two *T. gondii* genotypes. Twenty-six (45.6%) strains belong to the clonal Type II lineage (these strains can be further divided into two groups based on alleles at locus Apico). Eight (15.7%) strains belong to the Type III lineage. The remaining 22 strains were divided into 11 atypical genotypes. These results indicate high parasite prevalence and high genetic diversity of *T. gondii* in lambs, which has important implications in public health. We believe this is the first in-depth genetic analysis of *T. gondii* isolates from sheep in the USA.

III. Isolation of *Toxoplasma gondii* from bottlenose dolphins (*T. truncates)* [46]

J. P. Dubey, P. Fair, N. Sundar, G. Velmurugan, O. Kwok, W. McFee, **D. Majumdar**, C. Su
J. Parasitol., 94(4), 2008, pp. 821–823

Toxoplasma gondii infection in marine mammals (like- Dolphins and Sea otters) is intriguing and indicative of contamination of the ocean environment and coastal waters with oocysts. *Toxoplasma gondii* was isolated from hearts of the 3 dolphins (*T. truncates)* by bioassay in mice. Genotyping of these 3 *T. gondii* isolates (designated TgDoUs1-3) with the use of 10 PCR-RFLP markers (SAG1, SAG2, SAG3, BTUB, GRA6, c22-8, c29-2, L358, PK1, and Apico) revealed 2 genotypes. Two of the 3 isolates have Type II alleles at all loci and belong to the clonal Type II lineage. One isolate has a unique genotype. This is the first report of isolation of viable *T. gondii* from *T. truncatus*.

IV. Transplacental toxoplasmosis in naturally-infected white-tailed deer: Isolation and genetic characterisation of *Toxoplasma gondii* from foetuses of different gestational ages [48]

J.P. Dubey , G. Velmurugan, J. Gill , N. Sundar ,O. Kwok , P. Thulliez , **D. Majumdar** , C. Su
International Journal for Parasitology 38 (2008) 1057–1063

Clinical toxoplasmosis is most severe in congenitally-infected hosts. However, there are no data on the rate of congenital transmission of *T. gondii* with respect to gestational age in any host during natural infection. To know more about that, study was conducted on transplacental toxoplasmosis in naturally-infected white-tailed deer- a common game animal. Viable *T. gondii* was isolated from foetuses of six naturally-exposed white-tailed deer in early pregnancy (45–85 days of gestation) from Iowa and foetuses of nine naturally-exposed white-tailed deer from Minnesota in mid-gestation (130–150 days) of a gestational period of 7 months. The 15 *T. gondii* isolates obtained from foetal deer were PCR-restriction fragment length polymorphism genotyped using polymorphisms at 10 nuclear markers including SAG1, SAG2, SAG3, BTUB, GRA6, c22–8, c29–2, L358, PK1 and an apicoplast marker, Apico. Five genotypes were revealed, including the clonal Type II and III lineages, and three non-clonal genotypes. It is very likely that these nonclonal genotypes were derived from genetic crosses and natural recombination among the three clonal Type I, II and III lineages. The Type III strains were found at a lower frequency (13%, 2/15) and the majority were clonal Type II strains (60%, 9/15), commonly found in humans in North America and Europe, suggesting the possible link of transmission from common game animals to humans. This is important in public health and would suggest that more attention should be paid in preparing meat from game animals for human consumption. DNA sequencing analysis of representative isolates at loci SAG2, c22–8, L358 and PK1 revealed that the three non-clonal genotypes are closely related to the clonal Type I, II and III lineages.

V. Isolation and characterization of viable *Toxoplasma gondii* isolates revealed possible high frequency of mixed infection in feral cats from St Kitts, West Indies [53]

J.P. Dubey , L. Moura, **D. Majumdar** , N. Sundar, G. Velmurugan, O. Kwok , P. Kelly , C. Su
Parasitology (2009), 136, 589–594.

Cats are essential in the epidemiology of *Toxoplasma gondii* because they are the only hosts that can excrete the environmentally resistant oocysts in nature. *Toxoplasma gondii* was isolated from tissue samples of 7 feral cats from St Kitts, West Indies. All 7 isolates were avirulent for mice. Genotyping of these 7 *T. gondii* isolates by 10 multi-locus PCR-RFLP markers, including SAG1, SAG2, SAG3, BTUB, GRA6, c22-8, c29-2, L358, PK1, and an apicoplast marker, Apico, revealed 4 genotypes, including clonal Type II, Type III and 2 unique

genotypes. The 2 unique genotypes were not identified from any of the isolates collected from a variety of hosts in South America including Brazil, Colombia, Chile, Costa Rica, Nicaragua and Guyana. Five of the 7 cats had infection with 2 genotypes, indicating high frequency of mixed infection in the cat population on the St Kitts island.

VI. A new atypical highly mouse virulent *Toxoplasma gondii* genotype isolated from a wild black bear in Alaska [54]

J. P. Dubey, C. Rajendran, L. Ferreira, O. Kwok, D. Sinnett, **D. Majumdar**, C. Su
J. Parasitol., 96(4), 2010, pp. 713–716

A new mouse virulent atypical *T. gondii* genotype was isolated from an asymptomatic black bear (*Ursus americanus*) from Alaska, USA. The isolate (designated TgBbUS1) was mouse virulent, mice inoculated with 1 oocyst died of acute toxoplasmosis. The restricted fragment length polymorphism using 10 markers revealed that the strain possessed an atypical genotype; type I allele at loci SAG1, (5'-3')SAG2, SAG3,C22-8, C29-2, L358, and Apico, type II allele at locus alt.SAG2, and type III allele at loci BTUB, GRA6, and PK1. DNA sequencing at intron loci EF1, HP2 and UPRT1 revealed that the TgBbUS1 is a divergent *T. gondii* strain. These results indicate that mouse virulent atypical *T. gondii* genotypes are also circulating in wildlife in the US.

From all these genotyping results and additional studies by our collaborator Dr. Dubey at USDA, a total collection of 948 *T. gondii* isolates were analyzed and a total of 140 different distinct genotypes have been identified (Fig. 3.2) (Table 3.2). These results indicated that the overall diversity of *T. gondii* population might be much higher than previously thought. Genotypes #1 and #3 (clonal type II) form the most frequently isolated lineage and account for 26% of all isolates. Genotype #2 (clonal type III), the second most frequently identified lineage, accounts for 12% of isolates. Genotype #10 (clonal type I) accounts for only 2% of isolates. The above three lineages are widely distributed, though the type I strain, is infrequently isolated worldwide. Genotypes # 4, 5, 6, 7, 8, 9, 13, 14, 15, 17 and 18 are identified from at least two continents. Alleles which are distinct from those of types I, II or III are designated as either unique-1 (u-1), unique-2 (u-2), unique-3 (u-3) or 4, 5, 6, etc.

Phylogeny and Virulence: Phylogenetic network reconstruction analysis of the 140 distinct genotypes identified by Mn-PCR-RFLP revealed three major clusters: (i) Type II and related (~26%) (ii)Type III and related (~12%) and (iii) several not well distinguished sub-groups. (Fig. 3.3) Mouse virulence was determined in a total of 36 genotypes and 20 of these were non-virulent and 16 were virulent in murine models. Most of the virulent genotypes were from South America. (Fig. 3.3) (Table 3.2)

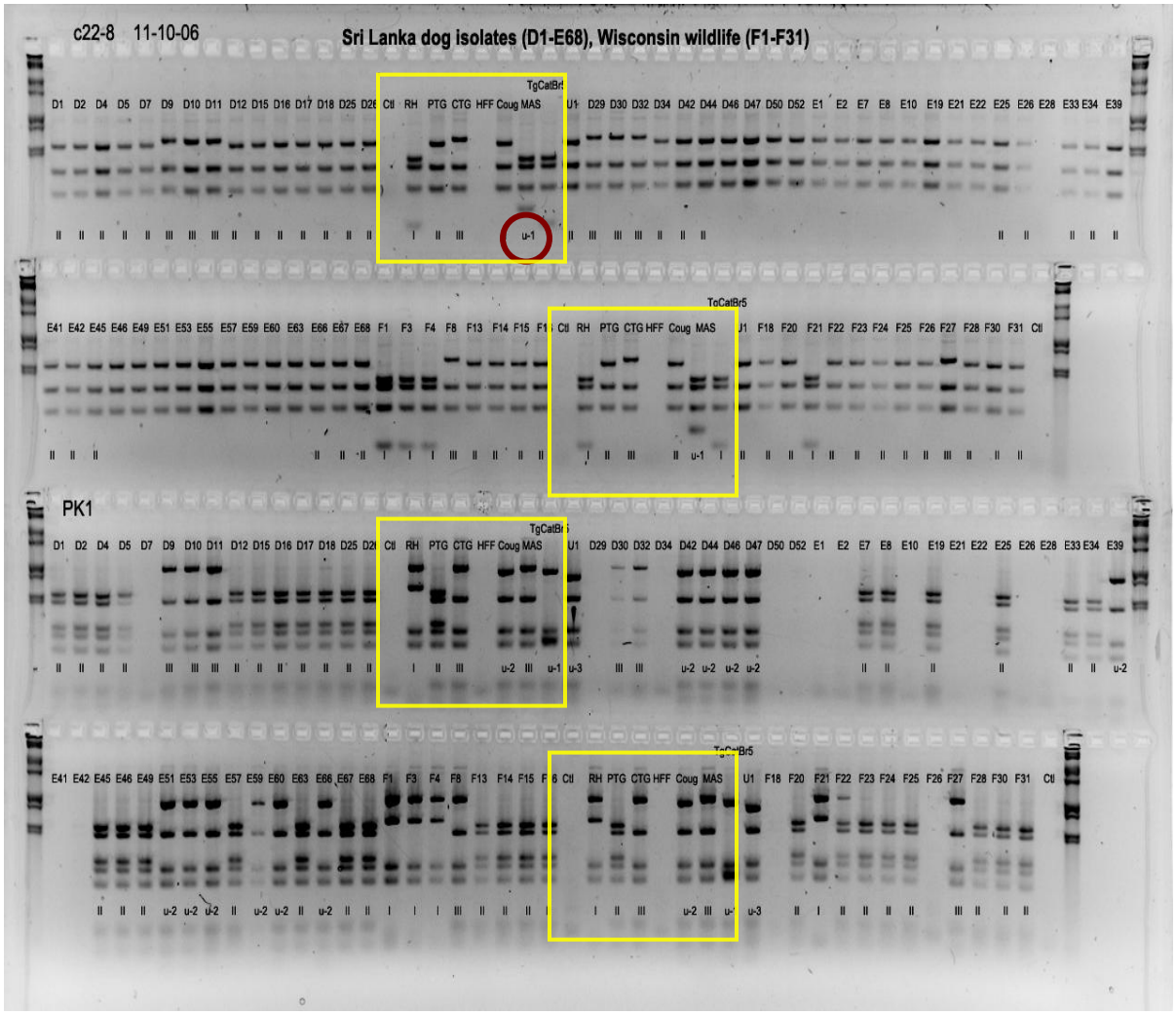


Fig. 3.2: Mn-PCR-RFLP genotyping of *T. gondii* isolates.
Reference strains and negative controls were always included in each row to monitor efficiency of PCR amplification and genotyping. The distinctive RFLP patterns of each isolate was compared to the reference type I, II and III strains to decide its genotype at that locus.

Table 3.1: Summary of Mn-PCR-RFLP genotyping of *T. gondii* isolates from my research publications

Sl. No.	Animal Host	Origin	No. of Isolates Studied	No. of Genotypes Identified
1	Sea Otter	N.America (U.S)	39	6
2	Lamb	N.America (U.S)	53	15
3	Bottlenose Dolphin	N.America (U.S)	3	2
4	White-tailed Deer	N.America (U.S)	15	5
5	Feral Cat	West Indies (St. Kitts)	7	4
6	Black Bear	N.America (U.S)	1	1

Table 3.2: Summary of 140 PCR-RFLP genotypes of *Toxoplasma gondii*

Genotype	Isolate	n=	SAG 1	5'+3' SAG 2	Alt. SAG 2	SAG 3	BTUB	GRA 6	c22-8	c29-2	L358	PK 1	Apico	Virulence	References
1	PTG (Type II)	143	II or III	II	II	II	II	II	II	II	II	II	II	Non	(Howe *et al.* 1995)[67]
2	CTG (Type III)	117	II or III	III	III	III	III	III	III	III	III	III	III	Non	(Howe *et al.* 1995)[67]
3	DEG	101	III or II	II	II	II	II	II	II	II	II	II	I	Non	(Ajzenberg *et al.* 2004)[4]
4	B41	53	II or III	II	II	II	II	II	II	II	I	II	I	Non	(Howe *et al.* 1995)[67]
5	WTD-1	34	u-1	II	II	II	II	II	II	II	I	II	I	Non	(Howe *et al.* 1995)[67]
6	BOF	33	I	I	I	III	I	II	u-1	I	I	I	I	Vir	(Howe *et al.* 1995)[67]
7	G622M	33	I	III	III	III	III	III	III	III	III	III	I	Non	(Howe *et al.* 1995)[67]
8	TgPgUs15(P89)	24	I	III	III	III	III	III	II	III	III	III	III	Non	(Howe *et al.* 1995)[67]
9	TgCtPRC4	23	u-1	II	II	III	III	II	II	III	II	II	I	Non	(Dubey *et al.* 2007)[43]
10	GT1 (Type I)	19	I	I	I	I	I	I	I	I	I	I	I	Vir	(Howe *et al.* 1995)[67]
11	TgCatBr01	15	I	I	II	III	III	III	I	III	I	II	III		(Su *et al.* 2006)[101]
12	IPP-BAT	13	I	III	III	I	I	III	II	III	III	I	III	Non	(Ajzenberg *et al.* 2004)[4]
13	TgCatStk7a	12	I	I	I	I	I	III	II	III	III	I	III		(Dubey *et al.* 2009)[53]

Table 3.2 continued

Genotype	Isolate	n=	SAG1	5'+3' SAG2	Alt. SAG2	SAG3	BTUB	GRA6	c22-8	c29-2	L358	PK1	Apico	Virulence	References
14	TgCatBr15	12	I	III	III	III	III	III	III	I	III	III	III	Vir-	(Su *et al.* 2006)[101]
15	CASTELLS	12	u-1	I	II	III	III	III	III	I	I	III	I	Non	(Ajzenberg *et al.* 2004)[4]
16	TgCkNi1	11	I	I	II	III	III	I	III	I	III	III	I	Non	(Dubey *et al.* 2006)[34]
17	MAS/ TgCkBr147	10	u-1	I	II	III	III	III	u-1	I	I	III	I	Vir	(Howe *et al.* 1995)[67]
18	TgCtPRC1	9	I	I	I	III	I	III	II	I	III	III	I	Non	(Dubey *et al.* 2007)[43]
19	TgCatBr05	9	I	III	III	III	III	III	I	I	I	u-1	I	Vir	(Su *et al.* 2006)[101]
20	TgDgSl4	9	u-1	II	II	III	III	II	II	III	II	u-2	I	Non	(Dubey *et al.* 2007)[35]
21	TgCatBr10	8	I	III	III	III	III	III	I	I	I	III	III		(Su *et al.* 2006)[101]
22	TgCkBr038	8	u-1	I	II	III	III	III	u-1	I	III	III	III	Vir	(Dubey *et al.* 2008)[47]
23	TgCkNi4	8	I	I	II	III	I	III	II	I	III	u-1	I	Non	(Dubey *et al.* 2006)[34]
24	TgCkCr4	6	I	I	I	I	III	I	I	I	I	I	III		(Dubey *et al.* 2006)[33]
25	TgCkGy08	6	I	III	III	I	III	III	III	III	III	I	I	Non	(Dubey *et al.* 2007)[39]
26	TgCatBr66	6	II or III	III	III	III	III	III	I	III	III	I	III	Non	(Pena *et al.* 2006)[90]
27	TgCkNi9	5	I	I	I	I	I	I	I	III	I	I	I	Vir	(Dubey *et al.* 2006)[34]

Table 3.2 continued

Genotype	Isolate	n=	SAG1	5'+3' SAG2	Alt. SAG2	SAG3	BTUB	GRA6	c22-8	c29-2	L358	PK1	Apico	Virulence	References
28	CAST	5	I	I	I	I	I	I	II	I	III	I	III		(Howe *et al.* 1995)[67]
29	TgCkBr114	5	I	I	II	III	I	III	II	I	III	III	I		(Dubey *et al.* 2008)[47]
30	TgCkGy07	5	I	III	III	I	III	III	III	III	III	I	III	Non	(Dubey *et al.* 2007)[39]
31	TgCkGy01	5	I	III	III	III	I	III	III	III	III	I	III	Non	(Dubey *et al.* 2007)[39]
32	TgRaW3	5	I	III	III	III	III	II	I	I	I	I	I		(Dubey *et al.* 2007)[42]
33	TgCkBr041	5	u-1	I	II	III	I	III	u-1	I	I	I	I	Vir	(Dubey *et al.* 2008)[47]
34	TgCatBr44	5	u-1	I	II	III	III	III	II	I	I	u-1	I	Vir-	(Pena *et al.* 2006)[90]
35	TgCkCr7	4	I	I	I	I	I	I	I	III	I	I	III	Vir	(Dubey *et al.* 2006)[33]
36	TgCkBr059	4	I	I	I	III	I	III	II	I	III	I	III	Vir	(Dubey *et al.* 2008)[47]
37	TgCkBr036	4	I	II	II	III	III	III	u-1	I	I	III	I		(Dubey *et al.* 2008)[47]
38	TgCtCo4	4	I	III	III	III	I	I	I	III	I	I	III	Vir	(Dubey *et al.* 2006)[34]
39	TgSoUs1	4	II or III	II	II	II	II	II	II	II	I	II	II		(Sundar *et al.* 2008)[103]
40	TgCkBr075	4	u-1	I	II	III	III	III	III	III	I	III	I	Vir	(Dubey *et al.* 2008)[47]
41	TgCkBr136	3	I	I	I	III	I	II	I	I	I	I	I		(Dubey *et al.* 2008)[47]

Table 3.2 continued

Genotype	Isolate	n=	SAG 1	5'+3' SAG 2	Alt. SAG 2	SAG 3	BTUB	GRA 6	c22-8	c29-2	L358	PK 1	Apico	Virulence	References
42	TgCatBr09	3	I	I	I	III	III	II	I	I	I	u-1	I		(Su *et al.* 2006)[101]
43	TgCkCr7	3	I	I	II	I	II	I	I	I	I	u-1	I		(Dubey *et al.* 2006)[33]
44	TgDgCo6	3	I	I	II	III	I	III	II	I	I	u-3	I	Vir	(Dubey *et al.* 2006)[34]
45	TgCkBr126	3	I	III	III	III	I	II	II	III	I	I	III		(Dubey *et al.* 2008)[47]
46	TgDgCo16	3	I	III	III	III	I	III	II	I	III	III	I		(Dubey *et al.* 2006)[34]
47	TgCatBr25	3	I	III	III	III	III	II	u-1	I	I	II	I		(Su *et al.* 2006)[101]
48	TgCkGy22	3	I	III	III	III	III	III	III	III	III	III	III		(Dubey *et al.* 2007)[39]
49	TgCatPr5	3	II or III	II	II	I	II	II	II	II	III	II	nd		(Dubey *et al.* 2007)[37]
50	TgCkNi45	3	II or III	III	III	I	III	I	III	III	III	III	III	Non	(Dubey *et al.* 2006)[34]
51	TgDgBr6	3	u-1	I	II	III	I	III	II	I	I	I	I	Vir	(Dubey *et al.* 2007)[39]
52	TgCkNi32	3	u-1	I	II	III	I	III	u-2	I	I	III	I	Non	(Dubey *et al.* 2006)[34]
53	TgDgBr15	3	u-1	I	II	III	III	III	II	I	I	III	I	Vir	(Dubey et al. 2007)[37]
54	TgWtdUs10	3	II or III	II	II	III	III	III	III	III	III	III	II		(Dubey et al. 2008)[44]
55	TgCatBr80	2	I	I	I	I	III	I	u-1	I	I	I	I		(Pena et al. 2006)[90]

Table 3.2 continued

Genotype	Isolate	n=	SAG 1	5'+3' SAG 2	Alt. SAG 2	SAG 3	BTUB	GRA 6	c22-8	c29-2	L358	PK 1	Apico	Virulence	References
56	TgCatBr45	2	I	I	I	III	I	II	u-1	I	III	I	I		(Pena et al. 2006)[90]
57	TgCkBr171	2	I	I	I	III	I	II	u-1	I	III	II	III		(Dubey et al. 2008)[49]
58	TgDgBr2	2	I	I	I	III	I	III	u-1	III	III	I	I		(Dubey et al. 2007)[37]
59	TgCkBr040	2	I	I	I	III	I	III	u-1	III	III	I	I		(Dubey et al. 2008)[47]
60	GUY002-2002-KOE	2	I	I	I	III	III	II	u-1	I	I	I	I		(Ajzenberg et al. 2004)[4]
61	TgCtCo5	2	I	I	II	I	III	III	III	I	III	III	I		(Dubey et al. 2006)[34]
62	TgCtCo3	2	I	I	II	III	I	III	II	III	I	u-2	I		(Dubey et al. 2006)[34]
63	TgCkBr013	2	I	I	II	III	I	III	II	III	III	III	I		(Dubey et al. 2008)[47]
64	TgCkBr019	2	I	I	II	III	III	III	I	III	I	II	I		(Dubey et al. 2008)[47]
65	TgCkBr089	2	I	I	II	III	III	III	u-1	I	I	u-2	I		(Dubey et al. 2008)[47]
66	TgCgCa1	2	I	I	II	III	III	III	u-1	I	I	III	I		(Dubey et al. 2008)[48]
67	TgCatBr76	2	I	II	II	III	II	II	II	u-1	I	u-2	I		(Pena et al. 2006)[90]
68	TgCkGy18	2	I	III	III	III	I	III	I	III	III	u-1	III		(Dubey et al. 2007)[39]
69	TgCkBr093	2	I	III	III	III	I	III	III	III	III	III	III		(Dubey et al. 2008)[47]

Table 3.2 continued

Genotype	Isolate	n=	SAG 1	5'+3' SAG 2	Alt. SAG 2	SAG 3	BTUB	GRA 6	c22-8	c29-2	L358	PK 1	Apico	Virulence	References
70	TgCkBr107	2	I	III	III	III	III	II	I	III	I	II	I		(Dubey et al. 2008)[47]
71	TgCkBr026	2	I	III	III	III	III	II	u-1	I	I	I	III		(Dubey et al. 2008)[47]
72	ROD-US	2	I	III	III	III	III	III	II	I	III	III	I		(Howe et al. 1995)[67]
73	TgShUs28	2	I	III	III	III	III	III	III	III	III	u-2	III		(Dubey et al. 2008)[45]
74	TgWtdUs8	2	II /III	III	III	I	I	I	III	III	III	I	I		(Dubey et al. 2008)[44]
75	TgCkBr048	2	II/III	III	III	III	II	II	II	III	II	II	I		(Dubey et al. 2008)[47]
76	TgCkBr155	2	u-1	I	II	III	III	III	II	I	I	III	III		(Dubey et al. 2008)[47]
77	TgCkBr141	2	u-1	III	III	III	III	III	u-1	I	I	III	I		(Dubey et al. 2008)[47]
78	TgCkBr169	1	I	I	I	I	I	I	u-1	I	I	III	III		(Dubey et al. 2008)[47]
79	TgDgCo13(50)	1	I	I	I	I	I	III	II	I	III	I	III		(Dubey et al. 2007)[38]
80	TgCatBr26	1	I	I	I	I	III	I	I	III	III	I	III		(Su et al. 2006)[101]
81	TgCkBr173	1	I	I	I	III	I	II	I	I	I	u-1	I		(Dubey et al. 2008)[47]
82	TgCkBr054	1	I	I	I	III	I	II	u-1	I	I	III	III		(Dubey et al. 2008)[47]
83	ENVL-2002-MAC	1	I	I	I	III	I	III	II	I	I	I	III		(Ajzenberg et al. 2004)[4]

Table 3.2 continued

Genotype	Isolate	n=	SAG 1	5'+3' SAG 2	Alt. SAG 2	SAG 3	BTUB	GRA 6	c22-8	c29-2	L358	PK 1	Apico	Virulence	References
84	PBr	1	I	I	I	III	I	III	II	III	III	I	III		(Khan et al. 2006)[76]
85	TgCatBr72	1	I	I	I	III	I	III	u-1	I	I	III	I		(Pena et al. 2006)[90]
86	TgCatBr50	1	I	I	I	III	III	II	u-1	I	I	II	I		(Pena et al. 2006)[90]
87	TgCkBr156	1	I	I	I	III	III	II	u-1	I	I	III	I		(Dubey et al. 2008)[47]
88	TgCkBr186	1	I	I	I	III	III	III	I	I	III	I	III		(Dubey et al. 2008)[47]
89	TgCkId1	1	I	I	I	III	III	III	II	I	III	I	III		(Dubey et al. 2008)[47]
90	TgBBeCa1	1	I	I	I	III	III	III	II	III	III	III	III		(Dubey et al. 2008)[48]
91	TgCkCr1	1	I	I	I	III	III	III	III	III	III	I	III		(Dubey et al. 2006)[33]
92	TgCatBr40	1	I	I	II	I	I	I	II	I	I	I	I		(Dubey et al. 2008)[47]
93	TgCkBr061	1	I	I	II	I	III	II	II	I	I	II	I		(Dubey et al. 2008)[47]
94	TgCkBr016	1	I	I	II	I	III	II	u-1	I	I	III	I		(Ajzenberg et al. 2004)[4]
95	GUY003-2002-MAT	1	I	I	II	I	III	III	I	I	I	II	I		(Dubey et al. 2008)[47]
96	TgCkBr109	1	I	I	II	I	III	III	II	I	I	III	I		(Ajzenberg et al. 2004)[4]
97	GUY001-2001-DOS	1	I	I	II	I	III	III	II	III	I	III	III		(Ajzenberg et al. 2004)[4]
98	RUB	1	I	I	II	I	III	III	II	III	I	III	I		(Ajzenberg et al. 2004)[4]

Table 3.2 continued

Genotype	Isolate	n=	SAG 1	5'+3' SAG 2	Alt. SAG 2	SAG 3	BTUB	GRA 6	c22-8	c29-2	L358	PK 1	Apico	Virulence	References
99	TOU021-2002-ALI	1	I	I	II	I	III	III	III	III	I	III	III		(Ajzenberg et al. 2004)[4]
100	GUY006-2003-BAS1	1	I	I	II	I	III	III	u-1	I	I	III	I		(Ajzenberg et al. 2004)[4]
101	TgCtCo15	1	I	I	II	I	III	III	u-1	I	III	III	I		(Dubey *et al.* 2006)[34]
102	TgCkNi35	1	I	I	II	III	I	III	II	I	I	u-2	I		(Dubey et al. 2006)[34]
104	TgCatBr34	1	I	I	II	III	I	III	II	I	III	u-1	III		(Su et al. 2006)[101]
105	TgCkBr143	1	I	I	II	III	I	III	u-1	I	I	III	I		(Dubey et al. 2008)[47]
106	TgDgBr18	1	I	I	II	III	III	II	u-1	III	III	III	I		(Dubey *et al.* 2007)[39]
107	TgCkBr037	1	I	I	II	III	III	II	u-1	I	I	II	I		(Dubey et al. 2008)[47]
108	TgCatBr57	1	I	I	II	III	III	II	u-1	I	I	III	I		(Pena et al. 2006)[90]
109	TgCkBr177	1	I	I	II	III	III	III	II	I	I	III	I		(Dubey et al. 2008)[47]
110	TgCkNg1	1	I	I	II	III	III	III	III	I	III	III	III		(Ajzenberg et al. 2004)[4]
111	TgCatBr64	1	I	I	II	III	III	III	u-1	III	I	III	I		(Pena et al. 2006)[90]
112	TgCatPr6	1	I	I	u-1	III	III	III	u-1	I	III	III	I		(Dubey et al. 2007)[37]
113	TgCkGy02	1	I	II	II	I	I	I	III	II	III	I	nd		(Dubey et al. 2007)[39]
114	TgCkBr166	1	I	II	II	III	II	II	II	nd	III	II	nd		(Dubey et al. 2008)[47]

Table 3.2 continued

Genotype	Isolate	n=	SAG1	5'+3' SAG2	Alt. SAG2	SAG3	BTUB	GRA6	c22-8	c29-2	L358	PK1	Apico	Virulence	References
115	TgCatPr9	1	I	III	III	I	III	III	III	I	III	I	I		(Dubey et al. 2007)[37]
116	TgCkBr130	1	I	III	III	I	III	III	III	III	III	III	I		(Dubey et al. 2008)[47]
117	TgCatBr41	1	I	III	III	III	I	III	II	III	III	III	III		(Pena *et al.* 2006)[90]
118	TgCatPr8	1	I	III	III	III	I	III	u-1	I	I	u-1	III		(Dubey *et al.* 2007)[37]
119	TgCatBr18	1	I	III	III	III	III	I	I	III	III	I	nd		(Su *et al.* 2006)[101]
120	TgCatBr20	1	I	III	III	III	III	II	u-1	I	I	u-1	I		(Su *et al.* 2006)[101]
121	TgCatBr67	1	I	III	III	III	III	III	I	I	III	III	III		(Pena *et al.* 2006)[90]
122	TgDgCo7	1	I	III	III	III	III	III	I	III	I	III	III		(Dubey *et al.* 2007)[38]
123	TgCkGy34	1	I	III	III	III	III	III	I	III	III	I	III		(Dubey et al. 2007)[39]
124	TgCatBr81	1	I	III	III	III	III	III	I	III	III	III	III		(Pena et al. 2006)[90]
125	TgCkBr008	1	I	III	III	III	III	III	II	III	I	u-1	I		(Dubey et al. 2008)[47]
126	TgCatBr06-20	1	I	III	III	III	III	III	II	III	III	u-2	III		(Su et al. 2006)[101]
127	B73	1	I	nd	I	III	III	II	u-1	I	I	u-1	I		(Howe et al. 1995)[67]
128	TgCtCo8	1	II or III	II	II	II	II	II	II	II	III	III	III		(Dubey *et al.* 2006)[34]
129	TgCkBr168	1	II or III	II	II	II	II	II	II	III	II	II	I		(Dubey et al. 2008)[47]

Table 3.2 continued

Genotype	Isolate	n=	SAG1	5'+3' SAG2	Alt. SAG2	SAG3	BTUB	GRA6	c22-8	c29-2	L358	PK1	Apico	Virulence	References
130	TgCatCa1	1	II or III	II	II	II	III	II	II	II	II	II	II		(Dubey et al. 2008)[48]
131	TgShUs32(0)	1	II or III	III	III	I	III	III	II	I	III	III	III		(Dubey et al. 2008)[45]
132	TgCkGh2	1	II or III	III	III	II	II	I	III	III	II	II	I		(Dubey et al. 2008)[47]
133	M7741	1	II or III	III	III	III	II	II	II	III	III	II	III		(Howe et al. 1995)[67]
134	TgCkBr178	1	II or III	III	III	III	III	I	III	III	III	III	III		(Dubey et al. 2008)[47]
135	TgCkBr045	1	u-1	I	II	III	I	III	II	III	III	I	III		(Dubey et al. 2008)[47]
136	TgCatBr38	1	u-1	I	II	III	III	III	II	I	III	III	I		(Pena et al. 2006)[90]
137	TgCkGh1	1	u-1	I	u-1	III	III	III	II	I	I	III	I		(Dubey et al. 2008)[47]
138	TgCkBr074	1	u-1	II	II	III	III	II	II	III	II	III	I		(Dubey et al. 2008)[47]
139	SOU	1	u-1	III	III	III	III	III	III	I	III	III	III		(Howe et al. 1995)[67]
140	TgCkNi27	1	II or III	II	II	III	III	III	II	II	III	III	I		(Dubey et al. 2006)[34]
141	TgCatStK1	1	II or III	III	III	III	III	III	II	III	III	I	III		(Dubey et al. 2009)[53]

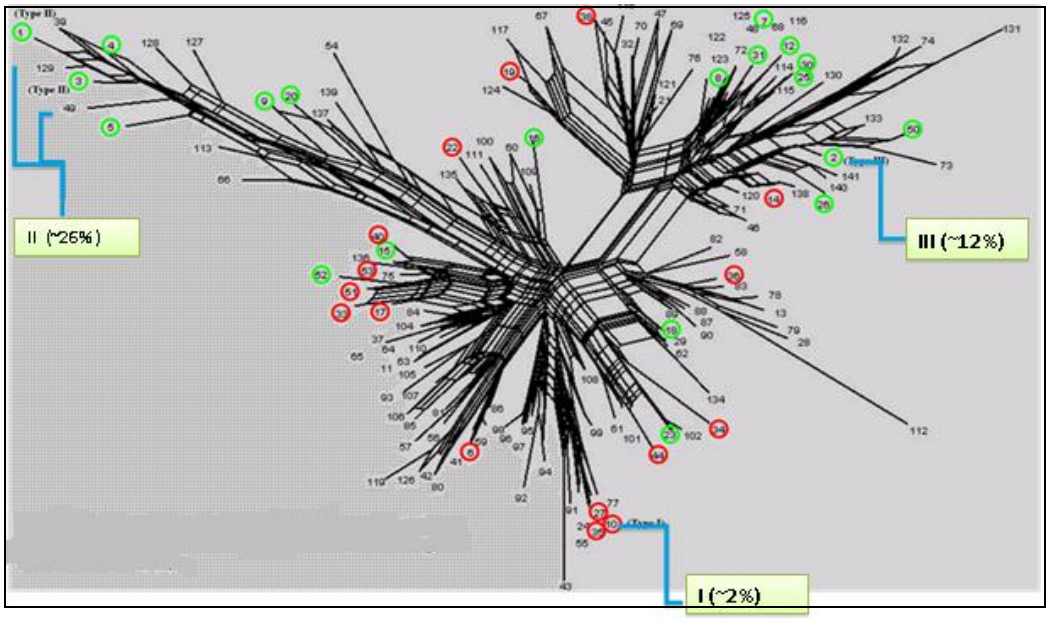


Fig. 3.3: Phylogenetic network of 140 *T. gondii* genotypes from 10 RFLP markers.
The *T. gondii* isolates were grouped into three major clusters: (i) Type II and related (~26%) (ii)Type III and related (~12%) and (iii) Type I (~2%) and several not well distinguished sub-groups. Twenty of these genotypes were non-virulent (green circles) and 16 (red circles) were virulent in murine models. Most of the virulent genotypes were from South American strains.

Chapter 4: Combined MS-RFLP-Intron Sequence Analysis of Distinct *T. gondii* Genotypes

Combined MS-RFLP-Intron Sequence Analysis of Distinct *T. gondii* Genotypes using 10 markers

4.1 Introduction

Three major genotyping methods (RFLP, microsatellites and multilocus intron sequencing) are used to study *T. gondii* population structure and epidemiology in different labs worldwide. Each of these methods has its own set of advantages and disadvantages. In the absence of a common reference platform it becomes very difficult to analyze and interpret the genotype data. In order to standardize studies and facilitate comparison among different laboratories, we conducted analysis of a composite data set of 133 genetically different *T. gondii* isolates by all three types of markers.

Microsatellites: Microsatellite (MS), another class of genetic markers[3,10] are short tandem repeats of 2-6 nucleotides. The DNA sequence length polymorphisms of these short nucleotide tandem repeats form the basis of microsatellite analysis. In *T. gondii*, the tandem repeats constituting the microsatellites occur 2–20 times and consist of as few as two nucleotides (di-nucleotide repeats). [3,4,10] Microsatellite polymorphisms arise due to size variation of these dinucleotide tandem repeats and consequently exhibit multiple alleles. This length polymorphism and allele sizing of microsatellite regions can be assessed with fluorescent primers after electrophoresis on an automatic sequencer. MS markers are sensitive, reliable and amenable to high-throughput analyses. They are highly polymorphic and have higher resolution than the housekeeping or antigen genes. But microsatellite markers can be prone to homoplasy as the number of repeats can expand and contract during replication. Micro-satellites are particularly useful tools for strain typing, [3,80] for epidemiological tracking and for individual identification of genetically closely related *T. gondii* isolates.[4]

Introns: Introns are the non-coding regions of DNA that serve as the selectively neutral marker loci. Intron sequencing within housekeeping or antigen genes is of particular value to phylogenetic studies involving *T. gondii* and other pathogens. Recently, intron sequencing analysis of *T. gondii* isolates has revealed otherwise previously unseen diversity in South

American isolates and has demonstrated that they cannot be adequately characterized based solely on microsatellites (homoplasy) or polymorphisms (RFLP) that discriminate among clonal lineages endemic to North America and Europe.[76,77]

4.2 Materials and Methods

Microsatellite and Intron Sequencing Analysis: 133 of the 140 newly identified distinct RFLP genotypes were further analyzed by multilocus microsatellites[3] and intron sequencing methods.[77,78] In total, 10 PCR-RFLP markers, 15 microsatellite markers (MS) and 4 intron markers were used for the analysis. The microsatellite analysis of the 133 genotypes by microsatellite markers was carried out in collaboration with Dr. Daniel Azjenberg's lab from University of Limoges, France. The 15 microsatellite marker loci used for this study include: *TUB2, W35, TgM-A, B18, B17,* M33, MIV, MXI, M48, M102, N60, N82, AA, N61 and N83. [110]

The intron markers selected for our study include a few commonly used introns: UPRT-1, UPRT-7, EF1 and HP2. The selected introns were PCR amplified, purified and then sequenced from one end by an automated sequencer. The primers used for the four intron markers are:

HP2: HP-Intron2-F,GACAGAAACACGCAGAGAAT and HP-Intron2-R, TAATCTTTGTTCCCATGCTT, which amplify a 846bp DNA fragment from *T. gondii*. The sequencing primer used is: HP-Intron2-Seq, ATAATACAGTCAGTTCCCTCGAT.

EF1: EF-Intron1-F,AAATGCACCCTTTTCTTAAA and EF-Intron1-R, CACATGAAGGTACACCAAAA, which amplify a 710bp DNA fragment from *T. gondii.* The sequencing primer used is: EF-Intron1-Seq, AAATTGTCCCGCCATCAG.

UPRT1: UPRT-F1,CCCGATATTCGACAAACGAC and UPRT-R1,GAGCCGTCTGCTTCATGAGC, which amplify a 555bp DNA fragment from *T.gondii*. The sequencing primer used is: UPRT-Intron 1-SeqF, TCAACCGAAGTTTGCTTTCC.

UPRT7: UPRT-Intron7-F1, TGGTCGTCGTCACTTGTTA and UPRT-Intron7-R1, GCAGCCTCACAACTAAAACT, which amplify a 760bp DNA fragment from *T.gondii*. The sequencing primer used is: UPRT-Intron 7-SeqF, TCTTGTTTGCTTTCCTCGGC.

Phylogenetic Analysis: The intron sequences were processed and aligned by using phylogenetic software package BioEdit. The Dendroscope program was used to analyze the composite MS-RFLP-Introns dataset of the common 133 *T.gondii* genotypes and the Neighbor- joining (NJ) trees were generated. Comparative topological analysis between the NJ trees was done using a web-based tool[88]: http://www.mas.ncl.ac.uk/~ntmwn/phylo_comparison/pairwise.html.
Global patterns of population structure and individual ancestry was inferred from the composite MS-RFLP-Introns dataset using a clustering approach with STRUCTURE software program.[55,56]

4.3 Results and Discussion

The microsatellite, RFLP and intron sequence analysis data was compiled and the results of individual and the combined analysis are tabulated below (Table 4.1) (Fig.4.6-4.9). Comparative topological analysis among the NJ trees of genotypes observed via multilocus microsatellite analysis and intron sequencing studies yielded a good correlation with resultant genotypes observed via multilocus PCR-RFLP on single-copy genes. Pair wise comparison of NJ trees between intron DNA sequences and composite MS-RFLP-Introns sequences revealed 47.3% similarity in terms of the overall topological score. Similar comparison between RFLP data and composite MS-RFLP-Introns sequences had 47.0% similarity in terms of the overall topological score.

Importantly, combining the multilocus microsatellites, RFLP and intron sequencing analysis results of the 133 representative genotypes into a composite phylogenetic network reconstruction gives a picture of the population structure. Instead of 3 clonal lineages, all *T. gondii* genotypes are clustered into 11 different haplogroups representing the major lineages (Fig 4.9). Three of these groups correspond to the previously recognized clonal lineages in North America and Europe (i.e. I, II and III). Within the new groups, five of these are almost exclusively composed of South American strains and the South American strains are mostly clustered separately from those in the North, showing strong geographical partitioning. Compared to the clonal pattern seen in type II, all the other groups generally show more deeply branching phylogenies which

might indicate the presence of a greater extent of sexual recombination in those groups . This analysis serves as a framework for further detailed study at the sequence level, regarding molecular epidemiology and population genetics of *T. gondii*.

Demographically, there is a strong clonal population structure reflected in North America, Europe and Africa. Majority of *T. gondii* isolates collected from human disease cases and chronic animal infections from Europe, North America and parts of Africa show remarkably little diversity and have been classified into one of two clonal genetic lineages (Types II and III). On the other hand, there are abundant unique genotypes in South America (Fig 4.5). Several new genotypes are frequently identified from there and high genetic diversity is evident in South America. Limited diversity is observed in Asia and Australia, where only a few lineages were identified. Interestingly, in South America, both clonal Type I and III lineages are present, but type II is largely absent. In previous virulence experiments, *T. gondii* isolates from asymptomatic chickens from Brazil and many other South American strains were seen to be more pathogenic to mice than isolates from Europe or North America, irrespective of the genotype. Taken together, high frequency of mouse-virulent genotypes in South America and dominance of non-virulent genotypes (types II and III) in other regions suggest different biological characteristics among different geographical locations.

These 133 representative *T. gondii* genotypes were further analyzed using STRUCTURE, (a Bayesian clustering method for predicting population structure and individual ancestry), to provide an estimate of the founding ancestral populations that can collectively explain the current population structure, assuming admixture of the original genotypes.[68] The STRUCTURE analysis results predicts 4 major ancestral clusters implying all existing *T. gondii* haplogroups to be derived from these four original ancestral populations. Three of the four groups correspond to the three clonal lineages, type I, II and III consisting of strains like-GT1 (blue), PTG (green), and CTG (red) respectively. The fourth founder genotypic group comprises of strains like- MAS, and TgCatBr18 (yellow). (Fig 4.6) The detailed statistical calculations involved in selecting the right 'K' value (=number of ancestral populations) for the STRUCTURE analysis is included in the appendix. (Fig 4.7) Further sampling and analysis in future would help reveal more information about genome evolution and the origin of *T. gondii*.

Table 4.1: Summary of Microsatellite, RFLP and Intron Sequence Analysis for 133 isolates

Chrm		IX	II	X	VII a	XII	IV	IV	XI	Ia	VII a	Ib	XII	VI II	VII b	X	VIII	VIII	VIII	XII	IX	X	Ib	III	V	VI		IV	X	XI
Isolate	0rigin	TU B2	W 35	Tg M-A	B1 8	B1 7	M 33	MIV .1	MXI .1	M 48	M1 02	N6 0	N8 2	A A	N6 1	N8 3	SA G1	5-3SA G2	SA G2	SA G3	BT UB	GR A6	c2 2-8	c2 9-2	L3 58	P K1	Api co	H P2	EF 1	UP RT
B41	N.A/ USA	289	242	207	162	336	169	274	356	213	170	142	111	287	107	316	II or III	II	II	II	II	II	II	II	I	II	I	4	5	11
B73	N.A/ USA	289	242	207	160	336	169	274	356	213	190	142	111	267	101	310	II or III	II	II	II	II	II	II	II	III	III	III	4	5	11
BOF	E.W/ Belgium	291	248	205	160	342	165	274	354	227	166	147	111	273	89	306	I	I	I	III	I	II	u-1	I	I	I	I	3	1	32
CAST	N.A/ USA	291	242	205	158	342	167	276	356	211	168	147	119	279	87	306	I	I	I	I	I	I	II	I	III	I	III	3	1	1427
CASTELLS	S.A/ Uruguay	287	242	207	158	358	169	274	356	239	164	138	109	283	87	324	u-1	I	II	III	III	III	III	I	I	III	I	2	3	1211
CTG	N.A/ USA	289	242	205	160	336	165	278	356	215	190	147	111	269	89	312	II or III	III	III	III	III	III	III	III	III	III	III	1	2	11
DEG	E.W/ France	289	242	207	155	336	169	274	356	219	174	138	113	265	99	310	II or III	II	II	II	II	II	II	II	II	II	I	4	5	11
ENVL-2002-MAC	Barbade	291	242	205	160	336	165	278	356	213	190	142	111	277	87	312	I	I	I	III	I	III	II	III	III	I	III	1	1	11
G622M	Central Panama	289	242	205	160	336	165	278	356	221	190	145	111	279	87	314	I	III	III	III	III	III	III	III	III	III	I	1	2	11
GPHT	E.W/ France	291	248	205	160	342	165	274	354	229	166	147	111	283	91	306	I	I	I	III	I	II	u-1	I	I	I	I	3	1	32
GT1	N.A/ USA	291	248	209	160	342	169	274	358	209	168	145	119	265	87	306	I	I	I	I	I	I	I	I	I	I	I	4	1	67
GUY001-2001-DOS	S.A/ French Guiana	289	246	203	160	344	167	272	356	229	176	142	113	263	85	320	I	I	II	I	III	III	II	III	I	III	I	5	7	811
GUY002-2002-KOE	S.A/ French Guiana	289	246	203	160	337	165	274	356	209	172	136	111	251	109	312	I	I	II	I	III	III	III	I	III	III	I	5	1	523
GUY003-2002-MAT	S.A/ French Guiana	291	242	203	160	339	165	272	358	221	174	138	107	277	95	310	I	I	II	I	III	III	II	I	I	III	I	5	1	812
GUY006-2003-BAS1	S.A/ French Guiana	289	242	203	160	344	165	276	356	211	168	142	109	271	87	312	I	I	II	I	III	III	u-1	I	III	III	I	3	12	824
IPP001-2002-BAT	S.A/ French Guiana	291	242	205	162	336	165	278	356	213	164	142	109	267	89	312	I	III	III	I	I	III	II	III	III	I	III	1	1	11

Table 4.1 continued

Chrm		IX	II	X	VIIa	XII	IV	IV	XI	Ia	VIIa	Ib	XII	VII	VIIb	X	VIII	VIII	VIII	XII	IX	X	Ib	III	V	VI		IV	X	XI
Isolate	0rigin	TUB2	W35	TgM-A	B18	B17	M33	MIV.1	MXI.1	M48	M102	N60	N82	AA	N61	N83	SAG1	5-3SAG2	SAG2	SAG3	BTUB	GRA6	c22-8	c29-2	L358	PK1	Apico	HP2	EF1	UPRT
M7741	N.A/USA	289	242	205	160	336	165	278	356	215	190	147	111	267	91	312	II or III	III	III	III	III	I	III	III	III	III	III	1	2	11
MAS	E.W/France	291	242	205	162	362	169	272	358	221	166	142	111	332	95	338	u-1	I	II	III	III	III	u-1	I	I	III	I	7	3	24
PBr	S.A/Brazil	291	248	205	160	334	165	274	354	213	190	140	119	271	87	308	I	I	I	III	I	III	u-1	I	I	III	I	1	3	32
PTG	N.A/USA	289	242	207	158	336	169	274	356	215	174	142	111	265	91	310	II / III	II	II	II	II	II	II	II	II	II	II	4	5	11
ROD-US	N.A/USA	289	242	205	160	336	165	278	356	213	190	147	111	267	89	314	I	III	III	III	III	III	III	III	III	u-2	III	1	2	11
SOU	N.A/USA	289	242	205	158	336	165	278	356	225	174	142	111	259	89	312	II or III	II	II	III	III	III	II	II	III	III	I	1	2	11
TgBBeCa1	N.A/Canada	289	248	209	160	336	165	278	356	213	166	149	107	265	87	306	I	I	I	III	III	III	III	III	III	I	III	1	1	11
TgCatBr01	S.A/Brazil	289	242	205	160	342	165	278	358	233	164	147	111	316	89	308	I	I	II	III	III	III	I	III	I	II	III	1	3	24
TgCatBr03 (idP89)	S.A/Brazil	289	242	205	160	348	165	278	356	213	190	142	111	263	113	312	I	III	III	III	III	III	II	III	III	III	III	1	2	43
TgCatBr05	S.A/Brazil	291	242	205	160	362	165	278	356	237	174	140	111	265	89	314	I	III	III	III	III	III	I	I	I	u-1	I	1	2	24
TgCatBr06-20	S.A/Brazil	291	242	205	162	342	169	272	356	237	164	145	111	265	89	314	I	nd	I	III	III	II	u-1	I	I	u-1	I	2	2	210
TgCatBr09	S.A/Brazil	291	242	205	160	362	165	278	354	227	174	140	111	269	89	308	I	I	I	III	III	II	I	I	I	u-1	I	1	1	32
TgCatBr10	S.A/Brazil	291	242	207	160	360	165	278	356	229	174	140	105	263	91	314	I	III	III	III	III	III	I	I	I	III	III	1	2	43
TgCatBr15	S.A/Brazil	289	242	205	162	344	165	278	358	225	164	142	111	263	105	312	I	III	III	III	III	III	III	I	III	III	III	1	1	421
TgCatBr18	S.A/Brazil	291	242	207	160	338	169	272	358	229	164	142	111	263	89	308	I	III	III	III	III	II	u-1	I	I	u-1	I	2	3	24
TgCatBr20	S.A/Brazil	289	242	205	160	360	165	278	356	213	174	140	105	265	105	314	I	III	III	III	III	III	I	I	III	III	III	1	2	43
TgCatBr25	S.A/Brazil	291	242	207	160	338	169	272	358	229	164	142	111	263	89	308	I	III	III	III	III	II	u-1	I	I	II	I	2	3	24

Table 4.1 continued

Chrm		IX	II	X	VIIa	XII	IV	IV	XI	Ia	VIIa	Ib	XII	VIII	VIIb	X	VIII	VIII	VIII	XII	IX	X	Ib	III	V	VI		IV	X	XI
Isolate	0rigin	TUB2	W35	TgM-A	B18	B17	M33	MIV.1	MXI.1	M48	M102	N60	N82	AA	N61	N83	SAG1	5-3SAG2	SAG2	SAG3	BTUB	GRA6	c22-8	c29-2	L358	PK1	Apico	HP2	EF1	UPRT
TgCatBr26	S.A/Brazil	291	248	205	160	362	165	278	354	229	174	140	111	271	89	308	I	I	I	III	I	II	I	I	I	u-1	I	3	1	32
TgCatBr34	S.A/Brazil	291	248	205	160	338	169	272	356	245	164	136	111	316	87	314	I	I	II	III	I	III	u-1	I	I	III	I	2	2	210
gCatBr38	S.A/Brazil	291	242	205	162	362	165	278	356	221	166	149	111	261	93	336	u-1	I	u-1	III	III	III	II	I	I	III	I	1	3	43
TgCatBr40	S.A/Brazil	291	242	207	162	338	169	272	358	223	176	145	113	316	91	308	I	I	II	I	III	II	II	I	I	II	I	10	3	215
TgCatBr41	S.A/Brazil	291	242	205	160	342	165	278	354	231	166	149	111	263	91	308	I	III	III	III	I	III	u-1	I	I	u-1	III	1	1	32
TgCatBr44	S.A/Brazil	291	242	205	162	342	165	278	356	231	166	153	111	265	91	344	u-1	I	II	III	III	III	II	I	I	u-1	I	1	2	55
TgCatBr45	S.A/Brazil	291	242	205	160	348	169	272	354	237	166	147	111	269	89	306	I	I	I	III	I	II	u-1	I	III	I	I	2	1	32
TgCatBr50	S.A/Brazil	291	248	205	160	342	165	274	354	231	166	147	111	265	89	310	I	I	I	III	III	II	u-1	I	I	III	I	3	1	32
TgCatBr57	S.A/Brazil	289	242	203	164	338	169	272	356	219	180	142	105	289	81	332	I	I	II	III	III	III	II	I	I	III	I	2	3	164
TgCatBr64	S.A/Brazil	289	242	207	160	338	165	278	356	225	190	136	105	263	97	310	I	I	u-1	III	III	III	u-1	I	III	III	I	1	3	43
TgCatBr66	S.A/Brazil	289	248	209	160	336	165	278	356	211	190	147	111	267	89	306	II or III	III	III	III	III	III	I	III	III	I	III	1	1	11
TgCatBr67	S.A/Brazil	289	242	205	160	348	165	278	358	213	164	145	111	263	123	308	I	III	III	III	III	III	I	III	I	III	III	1	3	24
TgCatBr72	S.A/Brazil	289	248	205	160	342	165	274	354	231	164	147	111	271	89	308	I	I	I	III	III	II	u-1	I	I	II	I	3	1	32
TgCatBr76	S.A/Brazil	291	242	205	160	342	165	278	356	237	176	140	111	265	93	314	I	III	III	III	I	III	I	III	III	u-1	III	1	2	55
TgCatBr80	S.A/Brazil	289	248	209	160	342	169	272	356	233	166	142	123	265	87	306	I	I	I	I	III	I	u-1	I	I	I	I	2	1	67
TgCatBr81	S.A/Brazil	291	242	207	156	338	165	276	358	225	166	145	107	265	91	316	I	III	III	III	III	III	II	III	I	u-1	I	2	2	215
TgCatCa1	N.A/Canada	289	248	209	160	336	165	278	358	213	190	142	113	263	93	306	II or III	III	III	I	III	III	II	I	III	III	III	1	1	11
TgCatPr5	Puerto Rico	289	246	207	162	336	169	274	356	233	166	147	113	267	93	312	II or III	II	II	I	II	II	II	II	III	II	nd	5	2	11
TgCatPr6	Puerto Rico	291	246	209	162	336	169	274	356	213	168	142	113	267	87	306	I	II	II	I	I	I	III	II	III	I	nd	4	1	131
TgCatPr8	Puerto Rico	289	250	209	160	336	165	278	358	213	166	142	111	265	87	310	I	III	III	III	III	I	I	III	III	I	nd	1	1	11

Table 4.1 continued

Chrm		IX	II	X	VIIa	XII	IV	IV	XI	Ia	VIIa	Ib	XII	VIII	VIIb	X	VIII	VII	VII	XII	IX	X	Ib	III	V	VI		IV	X	XI
Isolate	0rigin	TUB2	W35	TgM-A	B18	B17	M33	MIV.1	MXI.1	M48	M102	N60	N82	AA	N61	N83	SAG1	5-3SAG2	SAG2	SAG3	BTUB	GRA6	c22-8	c29-2	L358	PK1	Apico	HP2	EF1	UPRT
TgCatPr9	Puerto Rico (Mona Island)	289	242	205	164	342	165	278	356	213	172	147	111	267	91	306	I	III	III	I	III	III	III	III	III	III	I	1	1	11
TgCatStK1	Saint Kitts	289	242	205	162	336	165	278	NA	213	164	147	111	265	89	310	II or III	III	III	III	III	III	III	III	III	I	III	1	2	11
TgCatStk7a	Saint Kitts	291	242	205	162	342	165	278	356	211	164	142	109	277	87	312	I	I	I	I	I	III	II	III	III	I	III	1	1	11
TgCgCa1 (COUG)	N.A/ Canada	289	242	205	158	336	169	274	354	219	174	151	119	259	79	332	I	II	II	III	II	II	II	u-1	I	u-2	I	9	10	1826
TgCkBr008 (CK215)	S.A/ Brazil (Sao Paulo)	289	242	203	156	342	169	278	358	229	170	147	105	265	91	312	I	III	III	III	III	III	II	III	III	u-2	III	1	2	11
TgCkBr013 (CK210)	S.A/ Brazil (Sao Paulo)	289	242	205	160	338	169	276	356	213	164	145	111	316	89	312	I	I	II	III	III	III	I	III	I	II	I	2	2	43
TgCkBr016 (CK197)	S.A/ Brazil (Sao Paulo)	291	242	205	160	362	165	278	356	229	164	136	113	265	91	314	I	I	II	I	III	III	I	I	I	II	I	1	2	24
TgCkBr019 (CK211)	S.A/ Brazil (Sao	289	242	207	160	342	165	278	358	223	164	136	111	318	87	314	I	I	II	III	III	III	u-1	I	I	u-2	I	1	3	225
TgCkBr026 (CK300)	S.A/ Brazil (Rio)	291	242	207	162	342	165	278	358	229	164	151	105	265	91	310	I	III	III	III	III	III	II	I	III	III	I	1	8	24
TgCkBr036 (CK309)	S.A/ Brazil (Rio)	291	246	207	162	358	169	276	358	221	164	142	105	263	91	314	I	II	II	III	III	III	u-1	I	I	III	I	2	2	99
TgCkBr037	S.A/ Brazil (Rio)	291	242	205	162	338	165	276	356	231	164	138	105	289	105	330	I	I	II	III	III	II	u-1	I	I	III	I	2	3	28
TgCkBr038	S.A/ Brazil (Rio)	291	246	207	162	362	169	272	358	233	164	142	105	322	87	314	u-1	I	II	III	III	III	u-1	I	III	III	III	2	2	99
TgCkBr040 (CK312)	S.A/ Brazil (Rio)	291	248	205	160	342	165	274	356	235	166	147	111	269	89	308	I	I	I	III	III	II	u-1	I	I	I	I	3	1	24
TgCkBr041 (CK313)	S.A/ Brazil (Rio)	291	248	207	162	366	169	272	358	233	164	142	111	269	91	314	u-1	I	II	III	I	III	u-1	I	I	I	I	2	2	32
TgCkBr045 (CK323)	S.A/ Brazil (Rio)	291	242	207	164	342	169	272	356	229	172	142	105	320	81	310	u-1	I	II	III	III	III	II	I	III	III	I	2	8	43
TgCkBr048	S.A/ Brazil (Rio)	289	242	205	164	362	165	278	356	225	164	157	111	297	91	342	u-1	I	II	III	III	III	II	I	I	III	III	1	3	24
TgCkBr054 (CK342)	S.A/ Brazil (Rio)	291	248	203	160	342	165	274	354	221	166	142	111	269	89	330	I	I	I	III	I	III	II	I	I	I	III	3	3	32

Table 4.1 continued

Chrm		IX	II	X	VIIa	XII	IV	IV	XI	Ia	VIIa	Ib	XII	VIII	VIIb	X	VIII	VII	VII	XII	IX	X	Ib	III	V	VI		IV	X	XI
Isolate	0rigin	TUB2	W35	TgM-A	B18	B17	M33	MIV.1	MXI.1	M48	M1002	N60	N82	AA	N61	N83	SAG1	5-3SAG2	SAG2	SAG3	BTUB	GRA6	c22-8	c29-2	L358	PK1	Apico	HP2	EF1	UPRT
TgCkBr059	S.A/ Brazil	291	248	205	160	342	165	278	354	227	166	145	105	261	93	306	I	I	I	III	I	III	II	I	III	I	III	1	1	32
TgCkBr061	S.A/ Brazil (Rio)	291	242	205	162	338	165	276	356	235	176	140	119	289	99	330	I	I	II	I	III	II	u-1	I	I	III	I	2	3	28
TgCkBr074	S.A/ Brazil (Rio)	291	242	207	162	340	169	272	358	227	164	138	111	263	91	314	u-1	III	III	III	III	III	III	I	III	III	III	2	2	1114
TgCkBr075	S.A/ Brazil (Rio)	291	242	207	162	342	165	278	358	231	164	155	107	295	95	308	u-1	I	II	III	III	III	III	III	I	III	I	1	7	24
TgCkBr089 (CK351)	S.A/ Brazil (Rio)	291	246	207	162	362	169	278	358	227	164	142	107	326	87	314	I	I	II	III	III	III	u-1	I	I	III	I	2	2	99
TgCkBr093	S.A/ Brazil (Parana)	291	242	207	160	342	165	278	358	235	174	140	111	265	91	308	I	III	III	III	III	II	I	III	I	II	I	1	3	24
TgCkBr107	S.A/ Brazil (Para)	289	242	205	162	340	165	278	358	243	164	145	111	267	93	306	I	III	III	III	III	II	u-1	I	I	I	III	1	1	1114
TgCkBr109	S.A/ Brazil (Para)	291	246	203	156	336	167	276	356	215	174	145	111	265	101	316	I	I	II	I	III	III	II	III	I	III	III	3	9	518
TgCkBr114	S.A/ Brazil (Para)	291	242	205	160	358	165	274	356	233	194	142	121	293	NA	304	I	I	II	III	I	III	II	I	III	III	I	3	4	67
TgCkBr126	S.A/ Brazil (Rondonia)	289	248	205	160	342	169	278	354	213	166	142	111	269	105	310	I	III	III	III	I	II	II	III	I	I	III	1	1	43
TgCkBr130	S.A/ Brazil (Rondonia)	289	242	205	160	348	169	278	356	213	192	142	111	265	103	314	I	III	III	III	I	III	II	III	III	III	III	1	2	43
TgCkBr136	S.A/ Brazil (Rondonia)	291	242	205	160	342	165	278	354	229	166	140	111	271	91	308	I	I	I	III	I	II	I	I	I	I	I	1	1	32
TgCkBr141	S.A/ Brazil (Para)	291	248	209	160	344	165	278	356	209	166	142	113	281	95	306	I	I	I	I	I	I	u-1	I	I	III	III	6	1	1528
TgCkBr143	S.A/ Brazil (Para)	289	242	205	162	340	169	278	358	237	164	147	111	293	95	308	I	I	II	III	III	II	u-1	III	III	III	I	6	1	32
TgCkBr147	S.A/ Brazil (Rio Grande do Sul)	289	242	205	162	334	169	272	356	227	164	142	117	334	87	308	u-1	I	II	III	III	III	u-1	I	I	III	I	2	3	28
TgCkBr155	S.A/ Brazil(Rio rande)	289	242	205	160	334	167	276	356	213	190	140	119	267	87	308	u-1	III	III	III	III	III	u-1	I	I	III	I	1	3	28

Table 4.1 continued

Chrm		IX	II	X	VIIa	XII	IV	IV	XI	Ia	VIIa	Ib	XII	VIII	VIIb	X	VII	VII	VII	XI	IX	X	Ib	III	V	VI		IV	X	XI
Isolate	0rigin	TUB2	W35	TgM-A	B18	B17	M33	MIV.1	MXI.1	M48	M102	N60	N82	AA	N61	N83	SAG1	5-3SAG2	SAG2	SAG3	BTUB	GRA6	c22-8	c29-2	L358	PK1	Apico	HP2	EF1	UPRT
TgCkBr165	S.A/ Brazil (Pernambuco)	291	242	205	162	342	165	278	356	213	164	142	109	279	87	312	I	I	I	I	I	III	II	III	III	I	III	1	1	11
Isolate	0rigin	TUB2	W35	TgM-A	B18	B17	M33	MIV.1	MXI.1	M48	M102	N60	N82	AA	N61	N83	SAG1	5-3SAG2	SAG2	SAG3	BTUB	GRA6	c22-8	c29-2	L358	PK1	Apico	HP2	EF1	UPRT
TgCkBr166	S.A/ Brazil (Pernambuco)	289	242	207	160	342	165	278	356	211	190	149	105	263	101	306	I	III	III	I	III	III	III	I	III	I	I	1	1	43
TgCkBr168	S.A/ Brazil (Rio Norte)	291	242	207	158	336	169	274	356	235	176	138	119	261	93	310	II or III	II	II	II	III	II	II	II	II	II	II	4	5	11
TgCkBr169	S.A/ Brazil (Rio Grande do Norte)	291	242	205	162	342	165	278	356	227	164	142	109	277	91	312	I	I	I	I	I	III	II	I	III	I	III	1	-9	43
TgCkBr171	S.A/ Brazil (Maranhao)	289	242	207	160	348	169	278	356	213	196	147	111	265	87	308	I	I	I	III	I	II	u-1	I	III	II	III	1	-9	11
TgCkBr173	S.A/ Brazil (Bahia)	289	248	205	160	348	169	278	354	213	194	149	111	275	89	308	I	I	I	III	I	II	u-1	I	I	III	III	1	1	43
TgCkBr177	S.A/ Brazil (Ceara)	291	242	207	166	334	165	278	356	239	168	147	105	NA	93	310	I	I	II	III	III	III	III	I	III	III	III	1	13	43
TgCkBr178	S.A/ Brazil (Ceara)	291	242	205	160	336	165	278	356	213	NA	142	109	299	101	312	u-1	I	II	III	I	III	II	III	III	I	III	1	1	43
TgCkBr186	S.A/ Brazil(Alagoas)	291	248	205	160	342	165	278	354	237	166	142	105	263	89	306	I	I	I	III	III	III	II	I	III	I	III	1	1	32

Table 4.1 continued

Chrm		IX	II	X	*VII a*	XII	IV	IV	XI	Ia	*VII a*	*Ib*	*XII*	*VI II*	*VII b*	*X*	VIII	VIII	VIII	XII	IX	X	Ib	III	V	VI		IV	X	XI
Isolate	0rigin	*TUB2*	*W35*	*Tg M-A*	*B18*	*B17*	M33	MIV.1	MXI.1	*M48*	*M102*	*N60*	*N82*	*AA*	*N61*	*N83*	SAG1	5-3SAG2	SAG2	SAG3	BTUB	GRA6	c22-8	c29-2	L358	PK1	Apico	HP2	EF1	UPRT
TgCkCr1	Central Costa Rica	291	248	209	160	348	NA	276	358	209	166	142	125	259	89	306	I	I	II	I	I	I	II	I	I	I	I	4	1	32
TgCkCr4	Central Costa Rica	291	248	205	160	348	167	274	358	209	166	NA	123	279	89	304	I	I	I	I	I	I	I	III	I	I	III	3	2	616
TgCkCr7	Central Costa Rica	293	246	209	160	352	NA	278	356	209	166	140	105	259	95	304	I	I	II	I	II	I	I	I	I	u-1	I	3	1	32
TgCkGh1	Africa / Western / Ghana	293	242	203	156	336	165	NA	NA	215	176	130	109	281	NA	306	u-1	II	II	III	III	II	II	III	II	III	I	8	6	1013
TgCkGh2	Africa / Western / Ghana	289	242	205	160	336	165	278	356	213	190	140	111	267	93	312	II or III	III	III	III	II	II	II	III	III	II	III	1	2	11
TgCkGy01	S.A/ Guyana	291	242	205	162	336	165	278	356	213	164	149	111	281	87	312	I	III	III	III	I	III	III	III	III	I	III	1	1	11
TgCkGy02	S.A/ Guyana	291	242	205	162	336	165	278	NA	213	164	142	109	265	103	312	I	III	III	I	I	III	II	III	III	I	III	1	1	11
TgCkGy07 (TgCkGr7)	S.A/ Guyana	289	242	209	162	336	165	278	356	233	164	147	109	267	87	306	I	III	III	I	III	III	III	III	III	I	III	1	1	55
TgCkGy08	S.A/ Guyana	289	242	205	162	336	165	278	356	237	164	149	109	269	87	312	I	III	III	I	III	III	III	III	III	I	I	1	1	11
TgCkGy22	S.A/ Guyana	289	242	205	162	336	165	278	356	233	164	151	111	277	87	306	I	III	III	III	III	III	III	III	III	III	III	1	1	55
TgCkGy34	S.A/ Guyana	289	242	205	160	336	165	278	356	213	174	140	111	265	89	316	I	III	III	III	III	III	I	III	III	III	III	1	2	43
TgCkNg1	Africa / Western / Nigeria	287	242	207	158	354	169	274	356	219	168	138	109	283	85	322	u-1	I	II	III	III	III	III	I	I	III	I	2	3	1211
TgCkNi1	Central Nicaragua	289	242	209	160	348	167	276	356	209	166	147	111	259	NA	304	I	I	II	III	III	I	III	I	III	III	I	4	1	11
TgCkNi27	Central Nicaragua	289	242	205	160	336	165	278	356	213	188	142	125	277	87	304	II or III	III	III	III	III	III	II	III	III	I	III	3	4	76
TgCkNi32	Central Nicaragua	291	248	205	160	358	165	274	358	209	192	140	115	263	87	304	u-1	I	II	III	I	III	u-2	I	I	III	I	3	2	67
TgCkNi35	Central Nicaragua	291	242	205	160	336	165	274	356	223	190	142	111	279	91	312	I	I	II	III	I	III	II	I	III	u-1	III	1	2	11

Table 4.1 continued

Chrm		IX	II	X	VIIa	XII	IV	IV	XI	Ia	VIIa	Ib	XII	VII	VIIb	X	VIII	VIII	VIII	XII	IX	X	Ib	III	V	VI		IV	X	XI
Isolate	0rigin	TUB2	W35	TgM-A	B18	B17	M33	MIV.1	MXI.1	M48	M102	N60	N82	AA	N61	N83	SAG1	5-3SAG2	SAG2	SAG3	BTUB	GRA6	c22-8	c29-2	L358	PK1	Apico	HP2	EF1	UPRT
TgCkNi4	Central Nicaragua	291	242	205	160	336	165	274	356	223	194	142	121	281	89	304	I	I	II	III	I	III	II	I	III	u-1	I	3	4	817
TgCkNi45	Central Nicaragua	289	242	205	160	336	165	278	356	213	190	147	111	273	87	304	II or III	III	III	I	III	I	III	III	III	III	III	3	4	11
TgCkNi9	Central Nicaragua	291	242	205	160	346	167	274	358	211	166	147	127	265	85	304	I	I	I	I	I	I	I	III	I	I	I	1	1	11
TgCtCo15	S.A/ Colombia (Bogotà)	291	242	205	160	336	165	276	356	223	166	142	121	279	87	304	I	I	II	III	I	III	II	I	I	u-2	I	3	4	67
TgCtCo3	S.A/ Colombia (Armenia)	291	242	205	162	358	NA	274	356	233	194	142	123	297	NA	306	I	I	II	III	I	III	II	III	III	III	I	1	1	67
TgCtCo4	S.A/ Colombia (Armenia)	291	248	209	160	336	165	278	358	215	166	147	121	263	93	304	I	III	III	III	I	I	I	III	I	I	III	3	4	76
TgCtCo5	S.A/ Colombia (Bogotà)	291	242	205	160	336	165	276	356	223	166	142	121	279	87	308	I	I	II	III	I	III	II	III	I	u-2	I	4	5	11
TgCtCo8	S.A/ Colombia (Bogotà)	289	242	207	158	336	169	274	356	211	174	142	115	267	103	304	II or III	II	II	II	II	II	II	III	II	II	I	3	4	76
TgCtPRC4	Asia / Eastern / China	293	242	211	160	336	169	274	354	215	172	145	123	281	93	308	u-1	II	II	III	III	II	II	III	II	II	I	4	6	1022
TgDgBr15	S.A/ Brazil	291	242	205	162	362	165	278	356	233	166	149	111	295	91	342	u-1	I	II	III	III	III	II	I	I	III	I	1	3	32
TgDgBr18	S.A/ Brazil	291	242	205	162	338	169	276	358	229	164	142	111	324	89	342	I	I	II	III	III	II	u-1	I	I	II	I	1	3	24
TgDgBr6	S.A/ Brazil	291	248	205	160	362	165	278	354	231	166	151	111	273	91	316	u-1	I	II	III	I	III	II	I	I	I	I	2	3	210
TgDgCo13(50)	S.A/ Colombia / Bogotà	289	248	205	160	342	169	274	358	209	166	140	125	281	87	304	I	I	I	I	III	I	I	III	III	I	III	3	4	76
TgDgCo16	S.A/ Colombia / Bogotà	291	242	205	160	358	165	274	356	221	190	142	121	265	115	306	I	III	III	III	I	III	II	I	III	III	I	-9	1	55

Table 4.1 continued

Chrm		IX	II	X	VIIa	XII	IV	IV	XI	Ia	VIIa	Ib	XII	VIII	VIIb	X	VIII	VIII	VIII	XII	IX	X	Ib	III	V	VI		IV	X	XI
Isolate	0rigin	TUB2	W35	TgM-A	B18	B17	M33	MIV.1	MXI.1	M48	M102	N60	N82	AA	N61	N83	SAG1	5-3SAG2	SAG2	SAG3	BTUB	GRA6	c2 2-8	c2 9-2	L358	PK1	Apico	HP2	EF1	UPRT
TgDgCo6	S.A/ Colombia / Bogotà	291	242	205	160	336	165	276	356	223	166	142	130	287	91	312	I	I	II	III	I	III	II	I	I	u-3	I	4	1	11
TgDgCo7	S.A/ Colombia / Bogotà	289	242	209	162	336	165	278	356	213	164	140	111	263	97	306	I	III	III	III	III	III	I	III	III	I	III	3	4	43
TgDgSl4	Asia / Southern / Sri Lanka	293	242	203	156	336	165	274	354	215	174	130	107	279	97	312	u-1	II	II	III	III	II	II	III	II	u-2	I	8	6	1013
TgPgUs15-a.k.a.P89	N.A/ USA (Iowa)	291	242	205	160	348	165	278	356	213	190	142	111	261	87	304	I	III	III	III	III	III	II	III	III	III	III	1	2	43
TgRaW3 = TgRaW2	N.A/ USA (Wisconsin)	291	242	205	160	362	165	278	354	229	174	140	111	265	91	312	I	III	III	III	III	II	I	I	I	I	I	1	1	24
TgShUs28	N.A/ USA	291	248	209	160	336	165	278	354	213	166	147	111	277	87	314	II or III	III	III	I	I	I	III	III	III	I	I	1	1	32
TgShUs32(0)	N.A/ USA	289	242	209	160	336	169	274	354	213	166	147	111	277	87	308	II or III	III	III	II	II	I	III	III	II	II	I	4	1	32
TgSoUs1	N.A/ USA	289	242	207	162	336	169	274	356	215	170	142	111	267	97	304	II or III	II	II	II	II	II	II	II	I	II	II	4	5	11
TgWtdUs10	N.A/ USA	289	242	205	160	336	165	278	356	215	190	151	111	259	93	304	II or III	II	II	III	III	III	III	III	III	III	II	1	5	11
TgWtdUs8	N.A/ USA	289	242	207	160	336	169	274	356	227	190	142	111	269	103	310	II or III	III	III	III	II	II	II	III	II	II	I	1	2	11
TOU021-2002-ALI	Africa / Eastern / Réunion	289	242	205	160	338	169	272	356	231	178	142	119	301	89	310	I	I	II	I	III	III	u-1	I	I	III	I	2	11	220
WTD-1	N.A/ USA	289	242	209	160	336	169	274	362	223	170	157	113	287	99	312	u-1	II	II	II	II	II	II	II	I	II	I	4	5	11

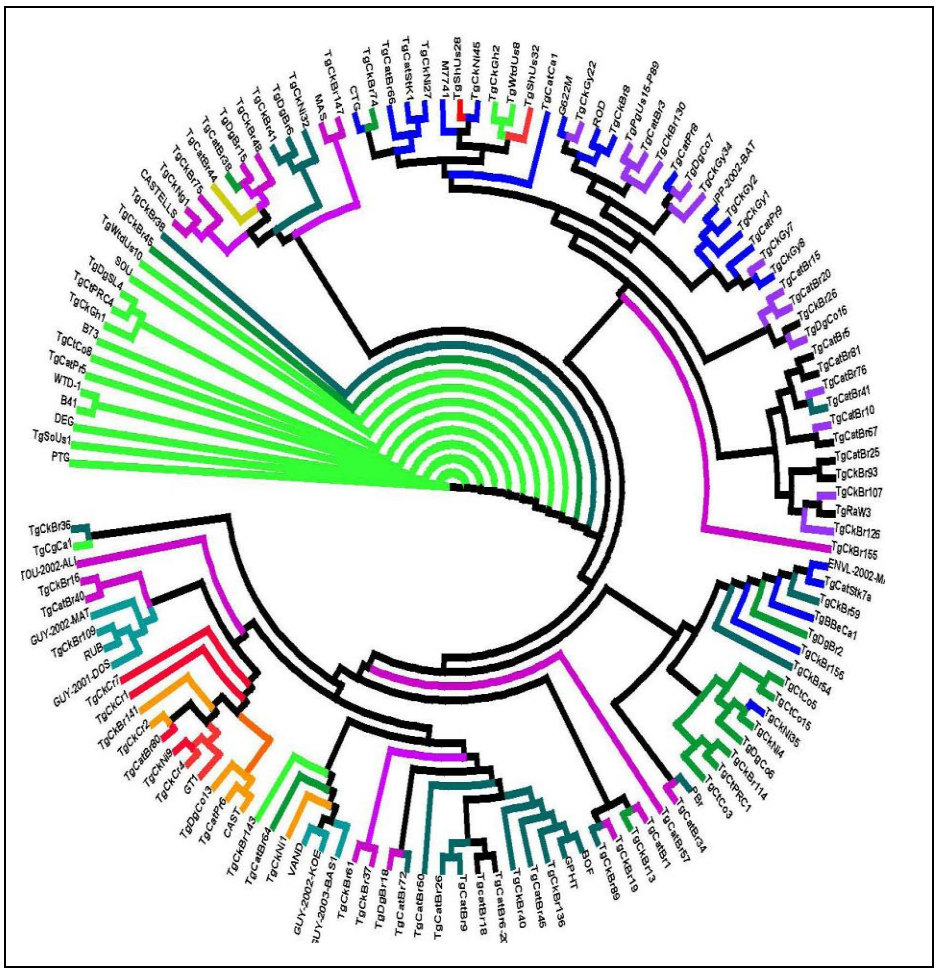


Fig. 4.1: Neighbor-joining (NJ) tree of RFLP markers of 133 *T. gondii* genotypes

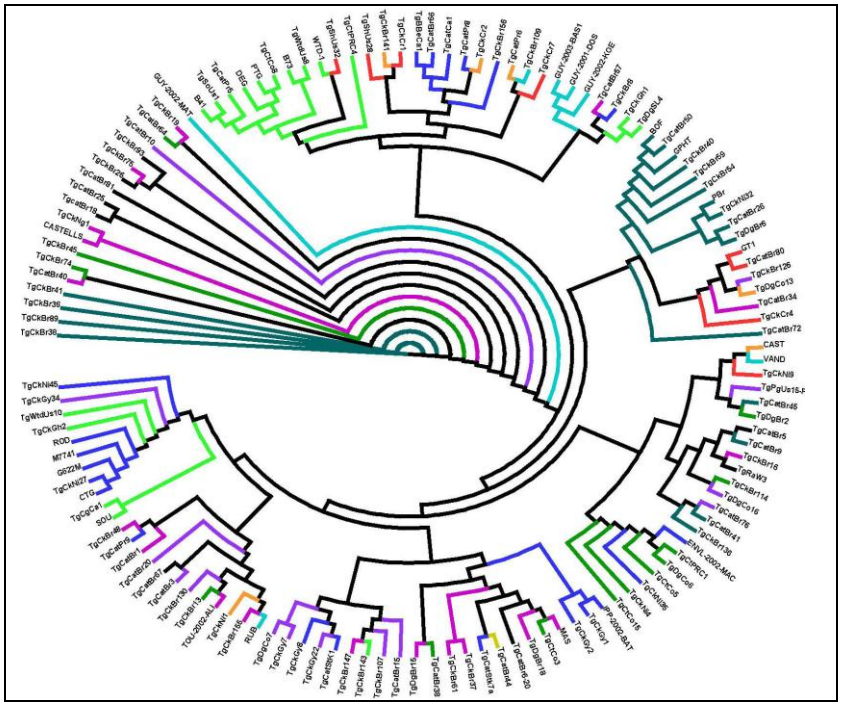


Fig. 4.2: Neighbor-joining (NJ) tree of MS markers of 133 *T. gondii* genotypes.

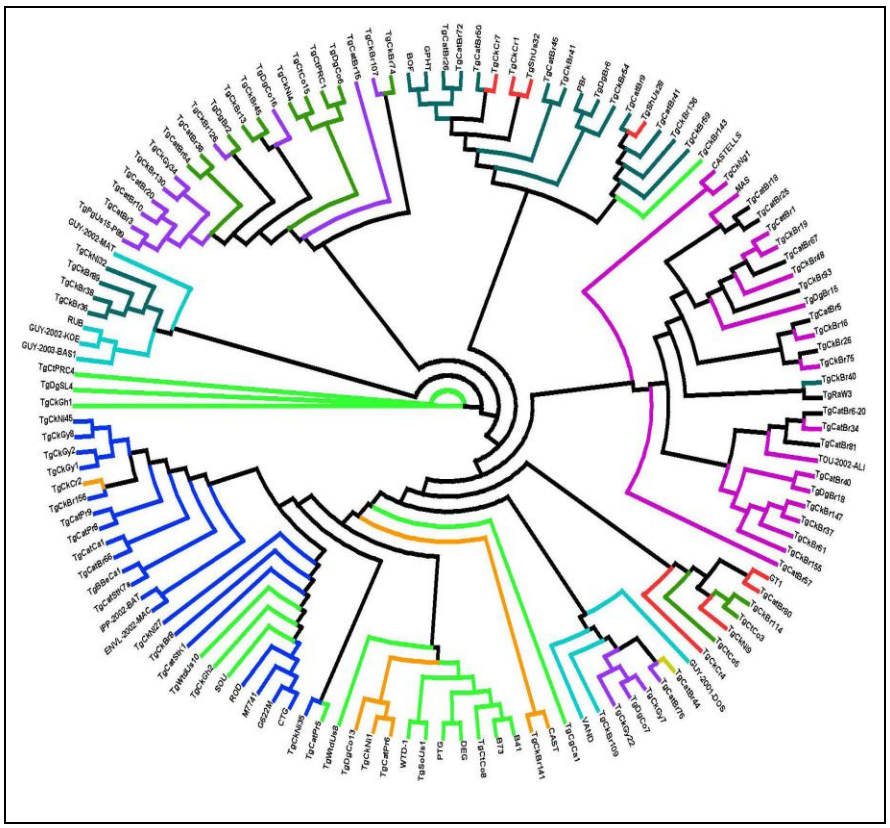


Fig. 4.3: Neighbor-joining (NJ) tree of introns of 133 *T. gondii* genotypes

Fig. 4.4: Combined NJ tree of MS-RFLP-Introns of 133 *T. gondii* genotypes.
All the *T. gondii* genotypes are clustered into 11 different haplogroups representing the major lineages. Each color represents a distinct haplogroup.

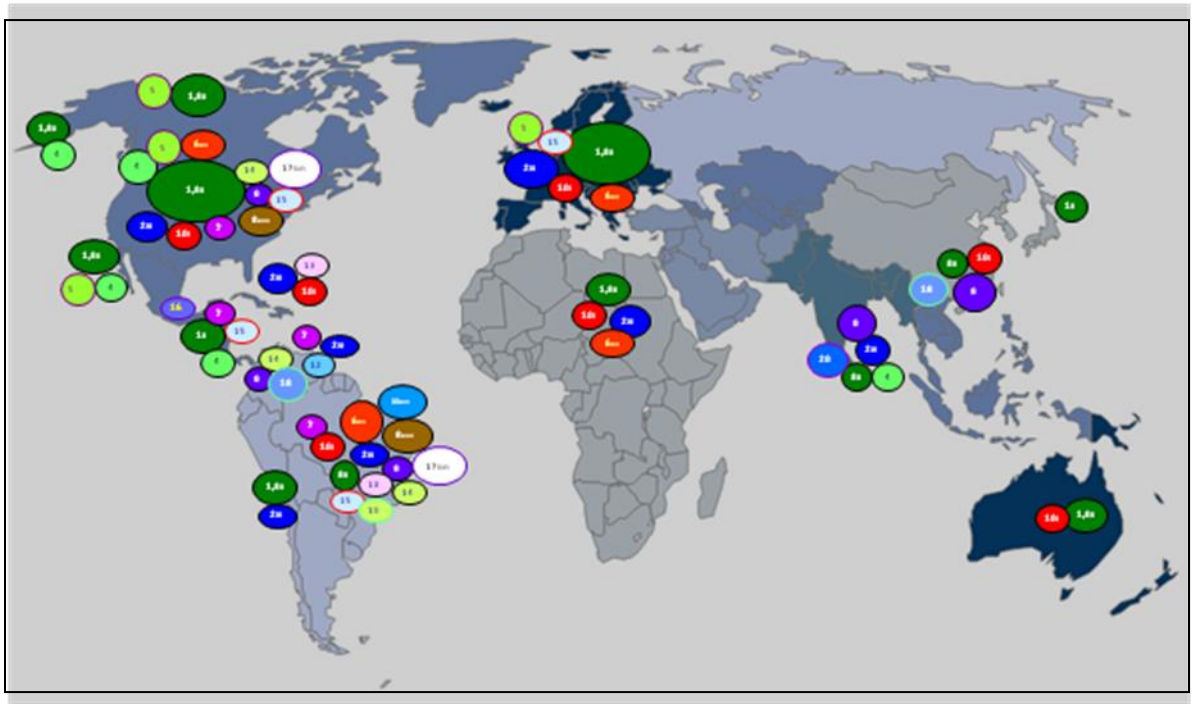


Fig. 4.5: Worldwide distribution of major RFLP genotypes.
The top 20 genotypes account for 78% of the isolates.

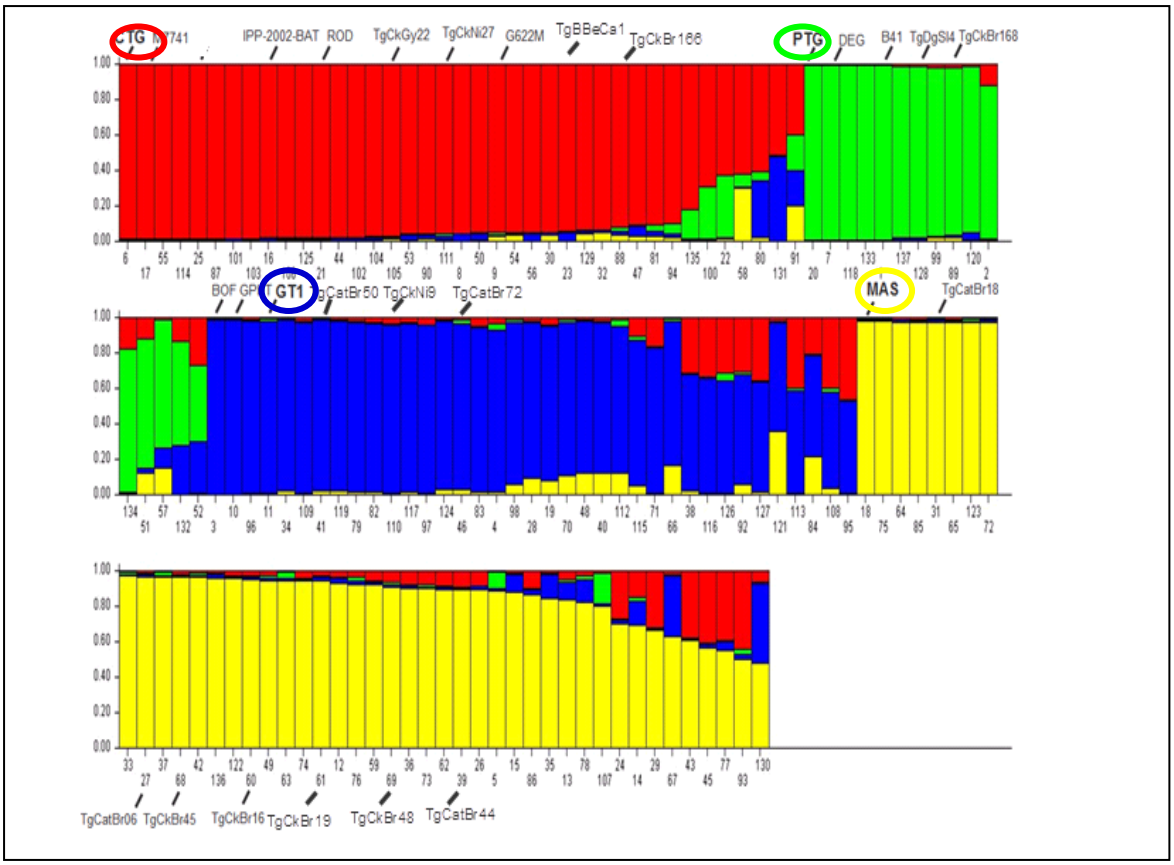


Fig. 4.6: Expanded view of STRUCTURE results at *K*=4.
STRUCTURE Analysis was performed with K = 4 ancestral populations using a Bayesian statistical approach to infer population structure under the assumption of admixture (41). Colors represent contribution from ancestral populations.

Chapter 5: Multilocus Sequence Typing (MLST) of Representative *T. gondii* Genotypes

Multilocus Sequence Typing (MLST) of Representative *T. gondii* Genotypes using 40 markers

5.1 Introduction

Genotyping *T. gondii* by the multilocus PCR-RFLP markers has generated invaluable information in revealing the parasite's diversity. But the prevalence of genetically more diverse strains in South America raises the question of whether they are distinct lineages or merely recombinants of the genotypes that are prevalent in the North. All the commonly used molecular methods of genotyping like- microsatellites or PCR-RFLP, underestimate the true rate of polymorphism and may misclassify variants owing to homoplasy or insufficient resolving power. For example, a high degree of polymorphism was observed at the *GRA6* locus by sequencing (nine allelic sequences from 30 strains), whereas the PCR-RFLP analysis only detected three groups.[57]

In contrast, sequence-based methods provide the best approach for detecting polymorphisms in new isolates or from previously sampled populations and also for resolving the extent of genetic variation among non-clonal strains. Thus, in the next part of our project, we carried out large-scale MLST studies in order to: 1) study the extent of genetic diversity among representative isolates; 2) survey the intensity of recombination and distinguish between diverged and recombinant lineages; 3) identify genes under strong selective pressure; 4) reveal information about the origin of *T. gondii* and its ancestral genomes and infer relationship among different genotypes.

Multilocus Sequence typing (MLST): The multilocus PCR-based sequence typing (MLST) method involves identification of all DNA sequence polymorphisms including the SNPs, small insertions and deletions of nucleotides in the sequence.[108] A variety of different loci have been used for sequence-based analysis, including both coding regions for housekeeping genes, antigens and selectively neutral introns.[77,108] Highly polymorphic antigens provide maximum resolution for detecting recent divergence within populations. In contrast, selectively neutral regions provide the best source of data to calculate the age of common ancestry between

different lineages and to predict common ancestry.[77] The obvious disadvantage of sequence-based typing is its increased cost and need for access to sophisticated technology. However, once the detailed population structure is known for a given region, more cost-effective typing methods can be developed to detect the major alleles (i.e. MS or RFLP typing). For example- the sequence information generated will be useful to select additional restriction endonucleases that recognize non-clonal Type I, II and III alleles, therefore eliminating the bias of these genetic markers. For phylogenetic studies too, MLST is preferred for its high resolution. Conventional PCR based MLST requires the samples to have a relatively high concentration of genomic DNA (~100 genome equivalents per PCR). Taking the Mn-PCR approach, it has been possible to generate quality DNA sequence data from samples containing as low as 10 genome equivalent.[102] Thus, sequence-based genotyping across multiple loci is the need of the hour.

5.2 Materials and Methods

Multilocus Sequence typing (MLST): Based on our preliminary PCR-RFLP genotyping results, large-scale multilocus PCR-based sequence typing (MLST) studies of these 140 newly identified genotypes has been carried out. A much larger set of 56 PCR-RFLP markers (2 to 5 loci per chromosome) localized among all 14 chromosomes has been employed to cover the entire *T. gondii* genome and to study the extent of recombination between clonal lineages. (Fig 5.1) Each of the 56 marker loci was PCR amplified, the PCR products were purified and then the target DNA was sequenced by an automated sequencer. (Fig 5.2) The complete list of primers used in this study for multilocus sequence typing (MLST) of representative *T. gondii* isolates has been summarized and attached to the appendix.(Table 5.2) This highly sensitive typing method has been used to achieve greater knowledge on epidemiology, population structure and molecular phylogeny of *T. gondii*.

The following 56 markers were used for the MLST studies:

Group1:

AK2, AK39, BSR4, PK1, CS3, SAG1, SAG2, BTUB, GRA6, SAG3, C22-8, IIC35, L358, VIIC373, RNAP1, 5rpoC2

Group 2:

ST12A, AK88, AK72, B10, AK11, ST2AN, VIIC356, AK127, AK112N, IMC2, MIC5, AK19, AK57, G11L, SAG4, ST5A, L366, MIC2, AK106, AK46

Group3:

AK3, MIC4, C29-2, ROP1, AK126, AK114, AK95, SRS4, AK15, AK150, AK26, AK102, XI-P89, Ia-P89, UPRT1, UPRT7, Hpintron2, EF-beta Intron1, GRA7, RNAP2

The MLST studies were done in conjunction with our collaborator's lab in china and of the 140 representative isolates, the following 30 *T. gondii* isolates were sequenced in our lab for each one of the afore-mentioned 56 loci:

TgCkBr165, TgCkBr166, TgCkBr168, TgCkBr169, TgCkBr171, TgCkBr173, TgCkBr177, TgCkBr178, TgCkBr186, TgCpBr1, TgCpBr25, TgCpBr26, TgCpBr27, TgGtBr1, TgGtBr5, TgShBr1, TgShBr3, TgShBr5, TgShBr12, TgOvBr3, TgOvBr7, TgOvBr9, TgOvBr11, TgOvBr15, TgPgBr14, TgPgBr24, GT1, CTG, and PTG.

Sequence Data Analysis: The total of ~5600 sequences generated from both our and our collaborator's lab were then pooled together and processed and aligned by using software package BioEdit. For each locus, the different allelic groups were identified from their individual phylogenetic trees. The allelic groups were sorted as per number of isolates present. Representative sequences from each allelic group were selected for generating a network tree via Splitstree4.[70] Using MEGA4 software, [104] columns with consensus sequences were deleted to identify and list only those regions possessing the SNPs among the representative sequences SNPs.

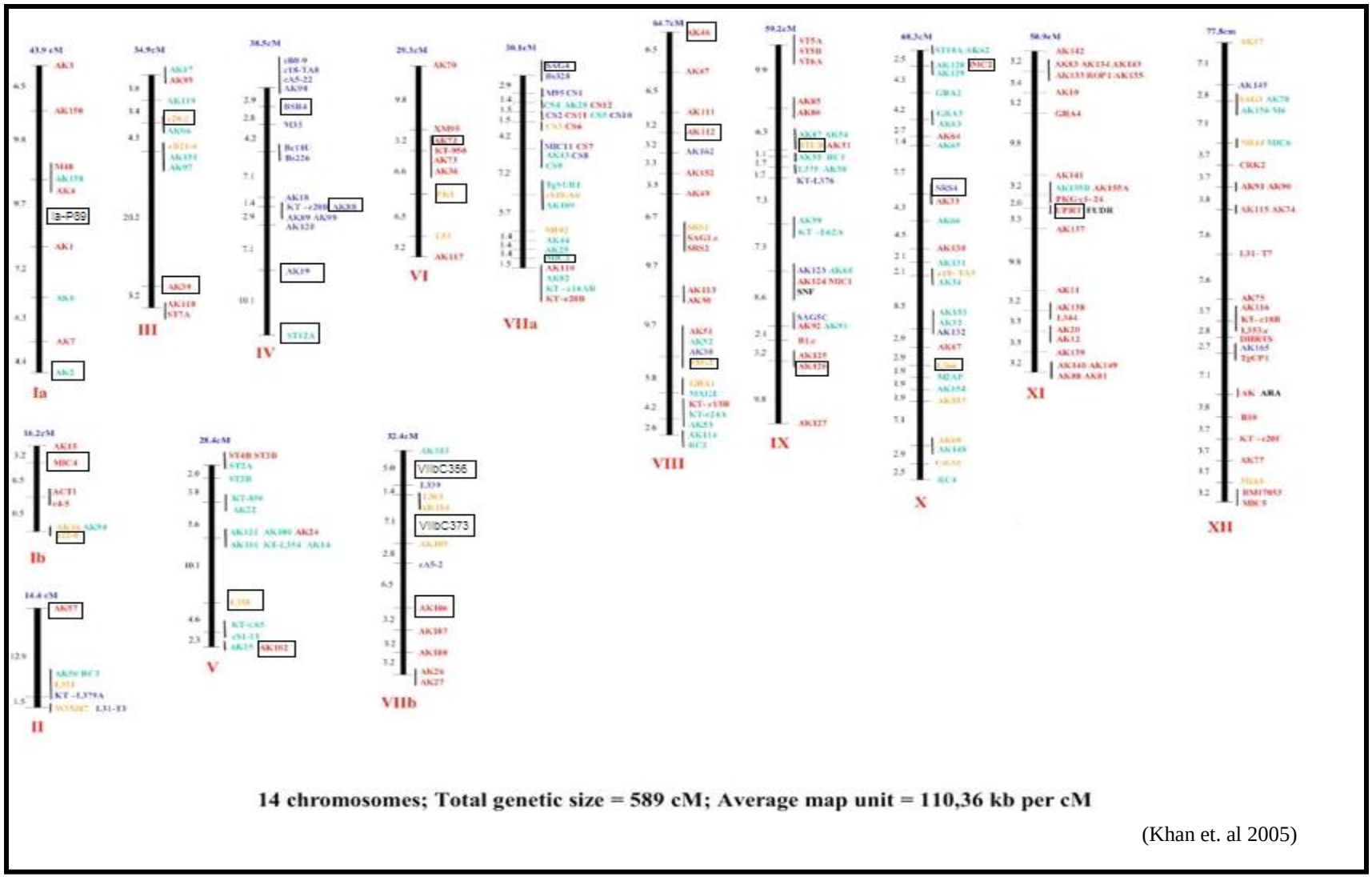


Fig. 5.1 : Genetic linkage map of *Toxoplasma gondii*.

T. gondii nuclear genome consists of 14 linear chromosomes and is ~65 Mb in size. The names in boxes are of some of the marker loci selected for our MLST study from each of the 14 chromosomes.

5.3 Results and Discussion

Multilocus DNA sequencing of 40 markers (out of total 56 markers) has been completed successfully and all the data generated by sequencing the 140 representative *T. gondii* genotypes using these 40 markers is summarized below. In most marker loci, (like- AK114, EF1, HP2, IMC2) the *T. gondii* strains are tightly clustered in star-shaped phylogenies with many short branches connected at the internal nodes. Such topologies are often inferred to represent a recent population expansion event from a common ancestor. (Fig. 5.3)

In the allelic distribution summary table, there isn't a lot of diversity and only four major alleles (a1, a2, a3, a4) were seen to be present among all clonal northern and even most non-clonal southern isolates suggesting a fairly recent origin of the parasite. (Fig. 5.4) For the three major clonal lineages, type I, II and III, three different distinct patterns emerge across all 40 loci/genome after being sorted as per their RFLP groups. Strains belonging to type II group seem to exhibit a clonal pattern and these results mirror the composite MS-RFLP-Introns sequence analysis results in which compared to Type II group, all the other groups show more deeply branching phylogenies. Chromosome Ia is not purely monomorphic, but has allelic variation; although not as much as the others. Marker loci such as ST12A and SAG2 located on chromosomes IV and VIII respectively, are highly conserved in almost all the strains. This might be just a random occurrence or these loci might be located in close proximity to certain genes that are important for the parasite's survival.[68] Overall, multilocus sequencing of representative *T. gondii* isolates confirmed a clonal propagation of some selected strains but also revealed recombination events more frequent than previously described.

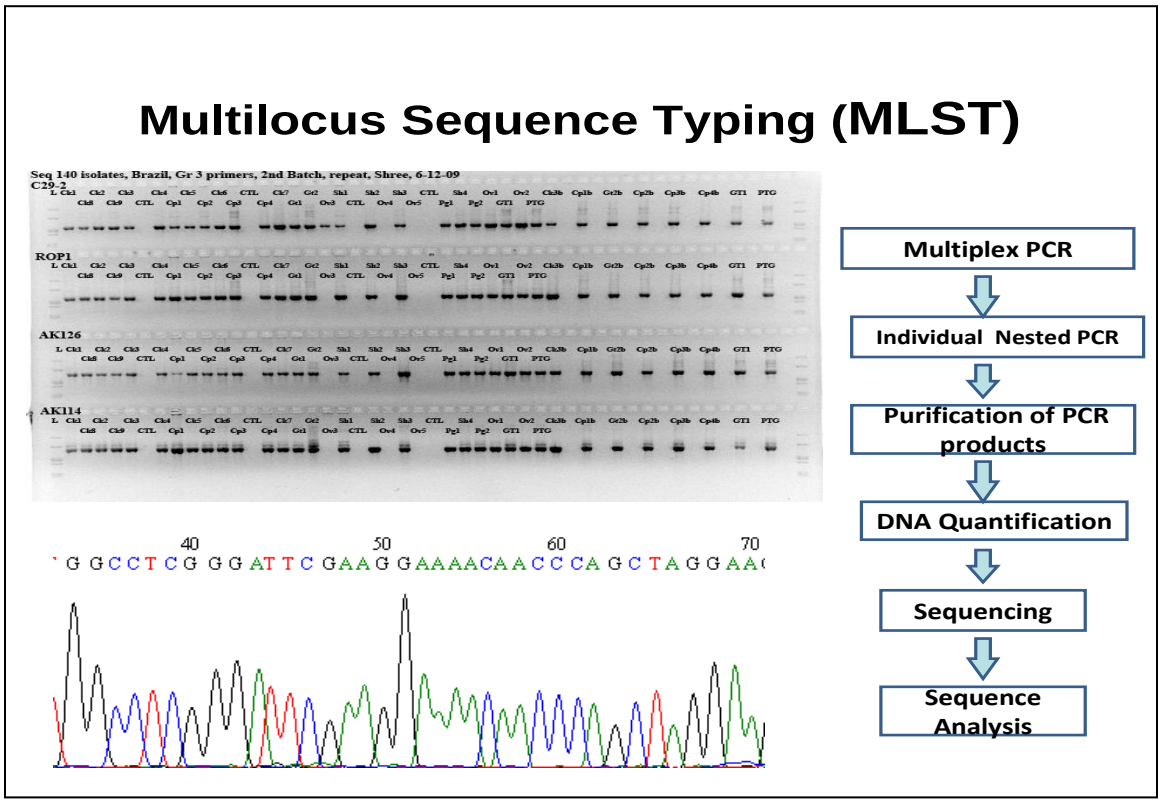


Fig. 5.2: Multilocus Sequence Typing (MLST) of representative *T. gondii* isolates

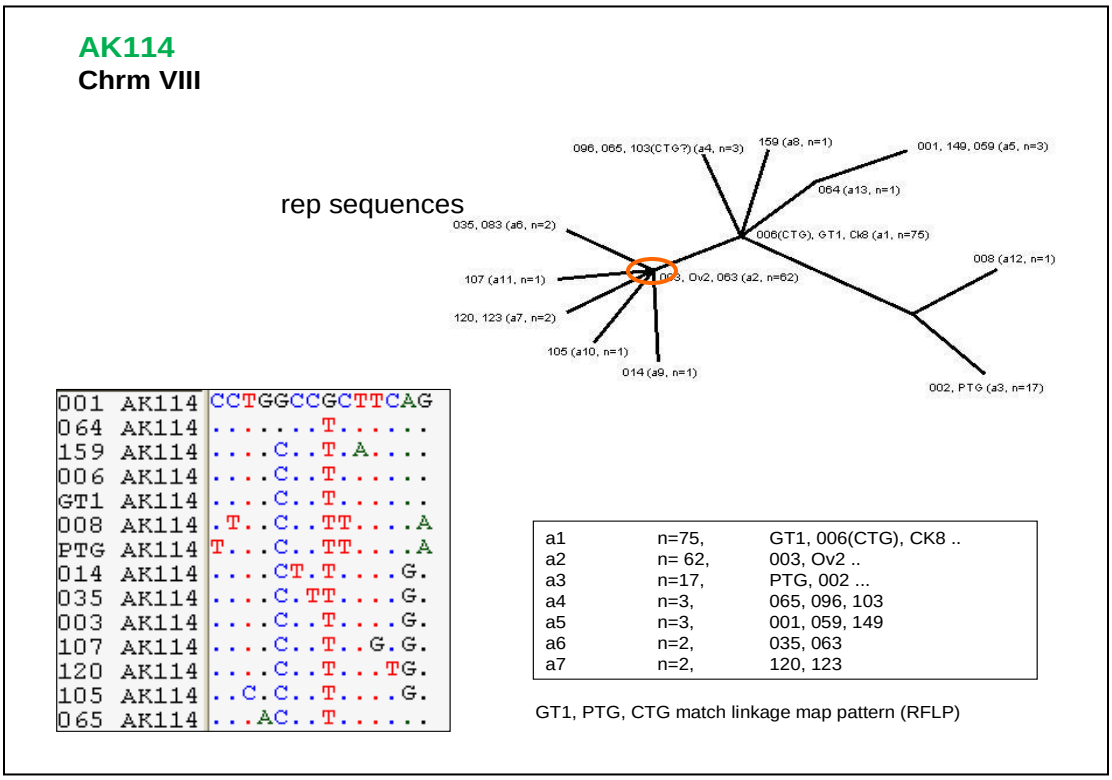


Fig. 5.3: *T. gondii* sequence polymorphism observed at each individual locus.
In most marker loci, (such as- AK114) the *T. gondii* strains are tightly clustered in star-shaped phylogenies

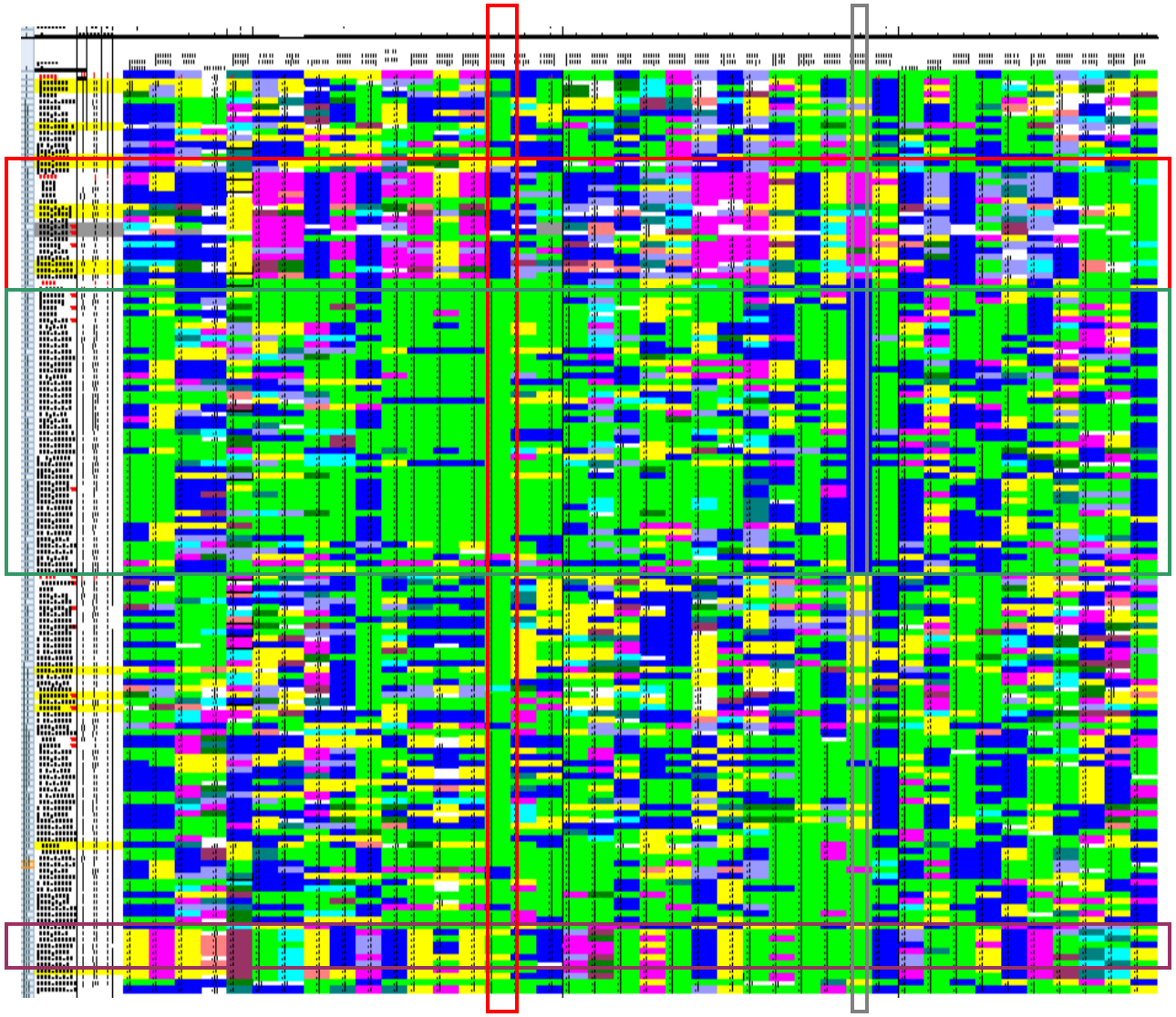


Fig. 5.4: Summary of distribution of allelic sequence data of representative *T. gondii* genotypes using 40 markers.

Only four major alleles: a1 (green), a2 (blue), a3 (yellow) and a4 (purple) were seen to be present among all clonal northern and even most non-clonal southern isolates.

Chapter 6: Conclusions and Future directions

Conclusions and Future Directions

6.1 Concluding remarks

Toxoplasma gondii possesses a well-characterized sexual cycle, yet the population structure of *T. gondii* in most regions reflects a high frequency of asexual replication. In Europe and North America, a large number of *T. gondii* isolates collected from human disease cases and chronic animal infections have shown remarkably little diversity and have been classified into one of three clonal genetic lineages (Types I, II, III) based on microsatellite typing, PCR-RFLP and intron sequencing studies with acutely virulent strains mostly comprising a single clonal type, type I. [3,20,96] Diverse genotypes sampled from wild animal isolates have been occasionally found.[103] Although genetic differences between the clonal lineages are small, they underlie considerable phenotypic differences especially in terms of virulence and infectivity.

While clonality clearly predominates in much of North America and Europe, additional sampling of animals or humans from a wider range of locations indicates that strains from South America and other geographical regions have more genetic variability than previously reported.[4] Most strains in South America are genetically distinct and have been believed to have evolved away from those in North America ~10^6 years ago. Despite having a more diverse population structure, South American strains still show pockets of clonality. This suggests an epidemic population structure of the parasite in Brazil, in which frequent genetic exchange has generated a variety of recombinants and a few successful clonal lineages have expanded into wider geographical areas. This is in sharp contrast to the clonal population structure in North America and Europe, where only three clonal lineages predominate and genetic exchanges among these lineages are rare.[3,20,67] Testing these models will require more information on modes of transmission in the wild and the global population structure scenario.

In addition, from the 11 major haplotype groups identified in *T. gondii* by composite MS-RFLP-Intron sequence analysis, 35 representative isolates have been selected for whole genome sequencing by JCVI/NIH NIAID. This will prove an important genome database for future studies on the biology *T. gondii*. These large-scale multilocus multichromosomal genotyping

and sequencing studies have also provided essential information to develop DNA barcode system for the identification of *T. gondii* in human and animal infection.

6.2 Future Directions

We still do not know the complete makeup of parasite populations in Africa, Asia, and other parts of the globe where *T. gondii* is abundant. Predicting the population structure of *T. gondii* in other parts of the world is uncertain and difficult given the markedly different population structure of *T. gondii* in North and South America. Limited sampling based largely on RFLP markers suggests that African populations are similar to those in Europe and North America.[81,109] These surveys focused primarily on domestic animals and in particular chickens, so they might be influenced by introduction of parasite strains from Europe along with importation of domestic animals. Further testing of feral animals will be necessary to properly define the population structure in these regions. In contrast, studies from Asia suggest predominance of genetically mixed strains,[37,99] yet the extent of genetic diversity in these regions has not been properly estimated. More sampling of both animal and human isolates and sequencing of introns and other loci from these isolates of these new regions is needed to capture the full genetic diversity and accurately explain the global population structure of this parasite.

The correlation, if any, between the parasite genotypes and the disease manifestation in human toxoplasmosis is another area of considerable interest and needs future investigation. Previous studies reported that certain strains were more frequently associated with a particular type of toxoplasmosis in human patients.[61,63,76] However, as there is no large scale epidemiological study to reveal the diversity of *T. gondii* genotypes both in human and animal populations, it is not clear if the bias of disease manifestations is due to the background genotypes in the environment where these patients reside, or the consequence of different biological traits that make certain genotypes more virulent in causing a particular type of human disease. The life cycle of *T. gondii* includes modes for sexual and asexual transmission; hence the population structure may vary dramatically in different localities. By understanding these patterns via more sampling studies, we can predict the risk of spread through the food chain and the potential for zoonotic infection. Defining the contribution of population structure to the spread of traits like immunogenicity and pathogenesis is highly significant to human health.

Modelling such relationships in a model parasite like *T. gondii* may also provide insight into other parasites of animals that pose risk to humans.

In the last few years, there has been considerable progress in population genetics and biodiversity of *Toxoplasma*. However, large-scale geographical genetic studies will be needed to understand the circulation of different genotypes across the world and to identify factors that facilitate clonal expansion of the type II strains. [97] Molecular-epidemiological studies on a global scale will help reveal the influence of both ancient (continental drift, plate tectonic formations of land and water barriers) or more recent phenomenon (domestication of cats and food animals, trade and migration together with cats and pests) on the genetic structure and transmission patterns of *T. gondii.*

Another important aspect of *Toxoplasma* research deals with the new emerging genotypes and the evolution of virulence. From the present series of experimental results and recent reports it is known that The *T. gondii* isolates from animals or human patients in South America are diverse and often bear unique genotypes suggesting the parasites underwent frequent sexual recombination. Also previous experimental genetic crosses between non-virulent type II and III strains have generated progeny with highly increased virulence in mice demonstrating that new biological traits can arise rapidly through random genetic reassortment of existing alleles. Therefore, it is necessary to identify factors that facilitate evolution of virulence in *T. gondii* and the new virulent genotypes that are out there. It is predicted that a multitude of new strains of *T. gondii* with altered phenotypic properties, some possibly highly virulent to human hosts, may circulate in the environment in South America and potentially cause severe disease in humans when contracted. This prediction is supported by a recent report showing that severe acute disseminated toxoplasmosis in immunocompetent patient was associated with new strains in that area.[17] These considerations are of more than theoretical interest because recombination of *T. gondii* in wild animals may lead to strains with newly acquired pathogenic mechanisms which can readily expand in the population, leading to emergent diseases. More virulence studies of the new atypical genotypes and further analysis of the genetic structure of populations will aid us in understanding the factors and mechanisms involved in the evolution of virulence as well as the underlying molecular basis of pathogenesis of this successful apicomplexan parasite.

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List of References

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Appendices

Appendix 1

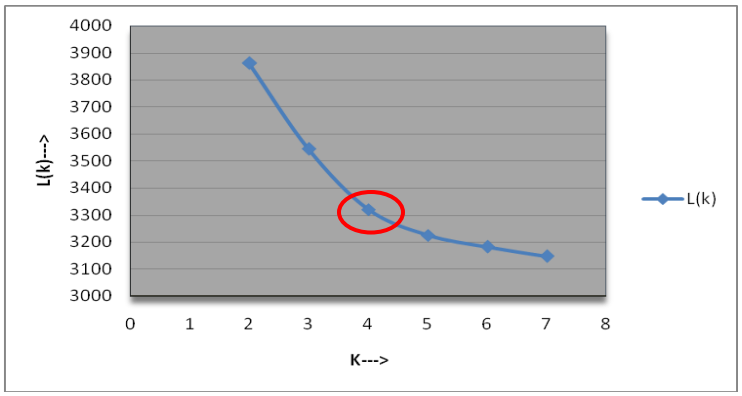
Statistics used to select 'K'

In order to estimate the number of ancestral populations using STRUCTURE, it is important to accurately estimate the 'K' value. The model choice criterion implemented to detect the true 'K' value is an estimate of the posterior probability of the data for a given 'K'. This value, called 'Ln P(D)' in structure output, is obtained by first computing the log likelihood of the data at each step of the Markov chain for each 'K'. The computed mean value of LnP(D) at each 'K' is termed as L(K) and this is used to identify the correct 'K' . The true value of 'K' corresponds to the L(K) value that represents the point of diminishing returns where the improvement in L(K)values starts to level off("knee of the graph").[56](Fig. 4.7) An alternative measure, denoted ΔK, is also used to accurately estimate 'K'. The maximal or peak value of ΔK indicates the preferred value of 'K', particularly when it is substantially larger than other choices with incorrect 'K' choices showing values much closer to zero.[55]

L (k) = | Mean Ln P(D)|
L' (k) = L(k) – L(k-1)
|L″(K)| =|L′(K + 1) − L′(K)|.
S.D = Standard Deviation of 'K'
Δ K = | L'' (K)/S.D|

K	Ln P(D)				
2	-3855	-3868	-3860	-3849	-3875
3	-3532	-3530	-3606	-3638	-3667
4	-3311	-3341	-3319	-3313	-3324
5	-3202	-3359	-3201	-3175	-3188
6	-3166	-3173	-3175	-3167	-3233
7	-3164	-3131	-3115	-3197	-3130

K	L(k)	L'(k)	L''(k)	S.D	ΔK
2	3861	-	-	-	-
3	3544	267	107	61.94	1.72
4	3321	273	177	11.99	**14.71**
5	3225	104	129	75.71	1.70
6	3182	43	35	28.32	2.81
7	3147	35	-	-	-



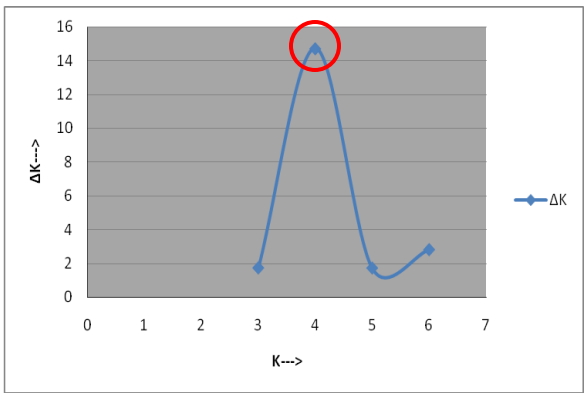


Fig. 4.7: True Estimation of 'K' for STRUCTURE analysis of *T. gondi*i isolates (K=4)

Appendix 2

Table 5.2: List of Primers used for MLST studies of *T. gondii* isolates

Chrm	Location (Me49)	Name	Sequence	bp
Ia	1838493- 1838869	AK2-F	CATGGCTTCGAGAACACTCA	609
		AK2-R	CGTCTGCTAGAATGCCAAGA	
		AK2-SqFx	CAAGTCTATCCTGAGCACGC	
	3051-3561	AK3-F	CACTCTCACCAGCCCTCTTC	612
		AK3-R	CATCGAGGTGTCATGTGAGG	
		AK3-SqR	GCCTGCAGTGTGTCGCTAT	
	169503- 170132	AK150-F	CCTTGCAGAGGCAACAGT	729
		AK150-R	ACCCAACGGAAGACAGAA	
		AK150-Seq	AGTTCTCGGGACCTTTCGG	
	1753947-1754461	Ia-P89-F	CCGCAAAGTAGTCGTCGTTA	675
		Ia-P89-R	AGCACTTTGTCGTTCGGACT	
		Ia-P89-SqFx	AGCCCTGTCAAACAACTGGA	
Ib	532892-533350	MIC4-F3	GTTGCCGTAGCAGGTAGCCAGTAGA	565
		MIC4-R3	CAGAGGAGCCTGTTCACG	
		MIC4-SeqN	GGGAACAGCGATAAAGCCATC	
	63505-63805	AK15-F	GCACAGAAATCGAGCAAA	600
		AK15-R	TAAAGGCGTGTACGTATC	
		AK15-SeqN	CGGACCCGAGCGGCAGTC	
	1852841-1853199	c22-8F	TCTCTCTACGTGGACGCC	521
		c22-8R	AGGTGCTTGGATATTCGC	
		c22-8SqF	AAGGATCGGGGAAAGTGTCT	
II	2077094-2077585	AK57-F1	CCTTCTTCACATTGTCTATGAAGGC	614
		AK57-R1	AGCACCAGCGTCGTAGTCCC	
		AK57-SqN	CGGCGGACACGAACGAGG	
	1865461-1866032	G11L-F	AGGACAATCCATACAGAAGAGGGGC	664
		G11L-R	GCAAAACGCTCGGGCAC	
		G11L-SeqN	AACGCTCGGGCACTCCTG	
	381058-381494	II-C35F	TTCATTCCCGAGTTCCTGAC	575
		II-C35R	GACACGCGCTACAAATCTGA	
		II-C35- SqFx	GTTCCTGACACTGCTCCCAG	
III	40126-40459	AK95-F	TTTCCTGCTCAGCTTCTCG	620
		AK95-R	GCGTTTGGCGGATCTTTC	
		AK95-Seq	AACCTGCGTCAGAGCAAGTCG	

Table 5.2 continued

Chrm	Location (Me49)	Name	Sequence	bp
	658336-658679	c29-2F	AGTTCTGCAGAGTGTCGC	446
		c29-2R	TGTCTAGGAAAGAGGCGC	
		c29-2 SqF	GGAGCATGTGGACACGTATG	
	2076286-2076641	AK39-F	TAGCTCACGTGGGAAAATTA	684
		AK39-R	AGCTCATAGAGGACCTTTGG	
		AK39-SqFx	GGAAATGCCAAAGCAAACAC	
IV	420099-420468	BSR4-F	CAAGGCTAACCGTAGCGGGT	715
		BSR4-R	CTCTTCAGGAACTTGGGGAA	
		BSR4-SqF	GGCACAGAAGTAACGCTGAA	
	1634429-1634976	AK88-F	AGGCGATAGCGGAATGAG	647
		AK88-R	CCGTTGCTGTAGCGTAAA	
		AK88-Seq	GCCTGAGTGATGGGTGGAAT	
	2474574-2475114	AK19-F1	ACAATGCGCAGGGACCGATCCTGGA	655
		AK19-R1	CCAGAGCTTGAAATTGGGCATCG	
		AK19-SqN	TGAAATCGCAAGCCGAAACA	
	1634682-1635151	HP Intron2-F	GACAGAAACACGCAGAGAAT	846
		HP Intron2-R	TAATCTTTGTTCCCATGCTT	
		HP Intron2-Seq1	ATAATACAGTCAGTTCCCTCGAT	
	2573895-2574391	ST12A-F1	AGAAGCAAACGGAGTGGT	593
		ST12A-R1	GACGCTCAGCTCAAAGACAA	
		ST12A-SeqN	GCTCAAAGACAACCGTGGC	
V	2189900-2190167	L358-F2	AGGAGGCGTAGCGCAAGT	418
		L358-R2	CCCTCTGGCTGCAGTGCT	
		L358-SqF	ATGTCCTCTTTCTGCCTTCG	
	3741194-3741667	ST2A-NF	GCTGTGGTTCGGTTTGTC	568
		ST2A-NR	AACTTCCTTCACTGTGGC	
		ST2A-Nseq	TGTGGCTAGGAGTCTGCATCAA	
	3038872-3039472	AK102-F	GACCTTCCTTCGCTAAAA	720
		AK102-R	CATCGGTTCTTCTGTTCTG	
		AK102-Seq	GAAAGAATACAAACGCAAGTCG	
VI	1037492-1037958	AK72-F	AATCTGCCTCGTCTTCCC	599
		AK72-R	TCCTGCCTTTCGCTTCTG	
		AK72-Seq	TTGTGCTGCAACGTCTGTCTTC	

Table 5.2 continued

Chrm	Location (Me49)	Name	Sequence	bp
	2652564-2653094	PK1-F	CGCAAAGGGAGACAATCAGT	903
		PK1-R	TCATCGCTGAATCTCATTGC	
		PK1-SqF	GGCACAATGGAAGACGATTT	
VIIa	263143-263654	SAG4-F	CCAACGCCGCTGAGACTA	611
		SAG4-R	TGAGCCTCAGTCCACGCA	
		SAG4-SeqN	ACGCCGCTGAGACTAACT	
	3515239-3515382	CS3-F	AGCGGATTTCCAACACTGTC	557
		CS3-R	CTGCTGCATTCACAAACTCC	
		CS3-SqF	CTTTTCGGATGTGGATTCGT	
	2568634-2569152	GRA7-F	TGACAGCAAACACTCACCG	843
		GRA7-R	CCAAAATGGCCCGACACG	
		GRA7-seqF	GTAGAGAGTCAGCTTCCGAGA	
	3758741-3759286	MIC2-F1	TGTGCCGAAGCTAGTGATTG	718
		MIC2-R1	GACAGTGCAGTCGCTCCAT	
		MIC2-SeqN	GCTTGGAGGCATCGTCACAGT	
VIIb	276959-277248	VIIbC356-F	TTCTGCGTCGGTTCTTCTCT	527
		VIIbC356-R	GCCTTTTCTGTGTCGACCTC	
		VIIbC356-Sq	TTGTTCTTGAGCGTTTCTTCTTCTC	
	2108906-2109334	VIIbC373-F	CCAGACATGGGCTTTGAAAT	529
		VIIbC373-R	CACAGTAAGCGTCGTTTCCA	
		VIIbC373-SqFx	GATTTTTGGAGTGGATCTGCA	
	2979700-2980107	AK106-F	TGAGGCACAGAAGAATGG	550
		AK106-R	GAAGAGGAGACACGAGGA	
		AK106-Seq	AGCGGCGGTTCACCTTTT	
	4438307- 4438888	AK26-F	TTCTGGAAATGCGTGGAC	657
		AK26-R	GCGACTTCTGCAACTCTAA	
		AK26-SeqN	TGGAAATGCGTGGACTTGCG	

Table 5.2 continued

Chrm	Location (Me49)	Name	Sequence	bp
VIII	110517-110996	AK46-F	GAAGCTCGGAGGACACCA	620
		AK46-R	TCAGGGTAACCAAACTAAGCAA	
		AK46-Seq	CGACCTCGCCAAAGAAGCA	
	1513670-1514148	AK112-NF	TTTCTGGAGCTGTGGTGTTC	574
		AK112-NR	CCTTCTTAGGACGGATAAGTCA	
		AK112-Nseq	GTTGTGCGCCATCGAGCTTAT	
	2662474-2662722	SAG1-S2	CAATGTGCACCTGTAGGAAGC	390
		SAG1-Rext	GTGGTTCTCCGTCGGTGTGAG	
		SAG1-AS2	TTATCTGGGCAGGTGACAAC	
	4753225-4753575	SAG2-Fa	ACCCATCTGCGAAGAAAACG	546
		SAG2-Ra	ATTTCGACCAGCGGGAGCAC	
		SAG2-SqF	TAGCTTTCAAGACCGCACCT	
	6746463-6746901	AK114-F	CAACTCCCACATTGACGC	568
		AK114-R	CTCAAGAGTCTACAGTTCCATTATG	
		AK114-Seq	ACTATGTTTTTCCGGCGCTCCTCAC	
IX	8090-8493	ST5A-F1	CTCCGCAGTTTGCTCACC	502
		ST5A-R1	CAGTGGACTCTGGACACCC	
		ST5A-SeqN	ATTATGGCACCGCAACAACC	
	974919-975136	BTUB -F	GAGGTCATCTCGGACGAACA	411
		BTUB -R	TTGTAGGAACACCCGGACGC	
		BTUB-SqF	GTGACAGCTTCGCCAGTGTA	
	5873341-5873742	AK126-F	CATGGCACGAGCTGCGGTAC	539
		AK126-R	GTTGTCATCGGTCAGTCG	
		AK126-Seq	CCTGGCATCGGACTTTCT	
	6142659-6143159	AK127BF	TCGTTTTGAGTTGCGTTATT	586
		AK127BR	CGAGAAATGGTGGTGAGA	
		AK127-Sq	CTCTCCCTTTTCTTTCCTTCCACTG	
X	468363-468862	IMC2-F	TGAGAACCTGGGAGGTAGGA	823
		IMC2-R	GCAGGAAGCAAACGTAGACC	
		IMC2-Sq	ACCAACGCCTTACAAGCAACT	
	2780508-2780991	SRS4-F	CGGCAAGGAAAGCAACCC	657
		SRS4-R	CTTCAAGCCCACCGTGTT	
		SRS4-Seq	GTTCCCAGGGTGTCCTCAAT	

Table 5.2 continued

Chrm	Location (Me49)	Name	Sequence	bp
	7215426-7215836	GRA6-For	GTAGCGTGCTTGTTGGCGAC	791
		GRA6-Rev	TACAAGACATAGAGTGCCCC	
		GRA6-SqR	GCACCTTCGCTTGTGGTT	
	1600933-1601379	EF1-Intron1-F	AAATGCACCCTTTTCTTAAA	710
		EF1-Intron1-R	CACATGAAGGTACACCAAAA	
		EF1-Intron1-Seq1	AAATTGTCCCGCCATCAG	
	6448244-6448480	L366-F	CGCGAGTTCGTGAGCATG	585
		L366-R	AAGTGGCAAGAGGGAGGA	
		L366-SeqN	GACGAAAGATGCGGGAAA	
XI	2709674-2709891	UPRT-F1	CCCGATATTCGACAAACGAC	555
		UPRT-R1	GAGCCGTCTGCTTCATGAGC	
		UPRT-SqF1	TCAACCGAAGTTTGCTTTCC	
	737520-737870	ROP1-F	CGTGACATATACTGCACTGAC	448
		ROP1-R2	CATCGTGAAACGTGTCATC	
		ROP1-Sq	GAGGCGGATTCATTAGTGGAGG	
	2712895-2713535	UPRT-Intron 7-F1	TGGTCGTCGTCACTTGTTA	760
		UPRT-Intron 7-R1	GCAGCCTCACAACTAAAACT	
		UPRT-Intron 7-SeqF	TCTTGTTTGCTTTCCTCGGC	
	3319755-3320125	AK11-F1	TTTGTGCGTACCGCTTCC	444
		AK11-R1	TACCCATGCTTCGCCCTC	
		AK11-SeqN	TGCGTACCGCTTCCCTACT	
	6369662-6370042	XI-P89-F	ATACTAGGGCGAGCAGGTGA	529
		XI-P89-R	TCCGACGAAAGCATGATACC	
		XI-P89-Seq	GCTGCTGTCTCCACTCTGGT	
XII	484231- 484761	SAG3-F	CACTCGCTCCGCTCTAAAAG	606
		SAG3-R	TCGACCTACACGCTGCAGTA	
		SAG3-SqRx	TCTTGTCGGGTGTTCACTCA	
	5437854-5438186	B10-F1	CCTTATGGGACGAGGTGT	494
		B10-R1	GTGTCTCGGCACGGGGTT	
		B10-SeqN	TATGGGACGAGGTGTGGATGGATTT	
	6712519-6713039	MIC5-F	GCGGTGGTCAGATTCCTCTA	714
		MIC5-R	GCCCAGTGTCGATAGCAAAT	
		MIC5-Sq	GCAAGCATTAGTGTCCTCGTC	

Table 5.2 continued

Chrm	Location (Me49)	Name	Sequence	bp
Plastid	12078-12285	rpoC2-F2	TTCCTCCTGTATGAAAAGTTCG	456
		rpoC2-R2x	CAAATGCTGGGTATTTAACACG	
		rpoC2-SeqF	TTTTGAACAAACTGTTCTTCCAC	
	8435-8993	RNAP-F1	AAAATAACGCGAAAAGATTCA	671
		RNAP-R1	TGTGGAAGATATGGAAATAAAGGA	
		RNAP-SeqR1	GCAGAAAACGCTGATTTACCTT	
	8443-8993	RNAP-F2	TCCTTTATTTCCATATCTTCCACA	640
		RNAP-R2	AAAACCTTTAGTATGAAACGGTGAA	
		RNAP-SeqF2	TTCCACATAATTTATCTCCAACTG	

Vita

Debashree Majumdar received her Master of Science (M.Sc.) Degree from the Orissa University of Agriculture and Technology, Orissa, India in 2001 with a concentration in Microbial Biotechnology. She then received a prestigious national research fellowship from the Department of Biotechnology, India and worked at the Central Institute of Fisheries & Aquaculture on a research project dealing with development of microsatellite markers in Indian major carp, *Labeo rohita*. In 2006, she began graduate studies in the Department of Microbiology at the University of Tennessee in Knoxville. In 2007, she was awarded the outstanding graduate teaching assistant award for her tutoring skills. During her graduate career, she has co-authored several research publications on *Toxoplasma gondii* population genetics in internationally acclaimed scientific journals. She has also participated in several meetings involving microbiology and *Toxoplasma gondii* including the 21st International Molecular Parasitology meeting at Woodshole, Massachusetts. She received her master's degree in Microbiology in December 2010 for her study on the population diversity of protozoan parasite *Toxoplasma gondii*. Following graduation, she plans to pursue her career in the field of scientific research.