REVIEW ARTICLE

Geographical patterns of *Toxoplasma gondii* genetic diversity revealed by multilocus PCR-RFLP genotyping

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SUMMARY

In recent years, an extensive collection of *Toxoplasma gondii* samples have been typed using a set of 10 PCR-RFLP genetic markers. Here we summarize the data reported until the end of 2012. A total of 1457 samples were typed into 189 genotypes. Overall, only a few genotypes dominate in the northern hemisphere, which is in stark contrast to the southern hemisphere where hundreds of genotypes coexist with none being notably dominant. PCR-RFLP genotype #1 (Type II clonal), #2 (Type III), #3 (Type II variant) and #10 (Type I) are identified globally. Genotypes #2 and #3 dominate in Africa, genotypes #9 (Chinese 1) and #10 are prevalent in Asia, genotypes #1, #2 and #3 are prevalent in Europe, genotypes #1, #2, #3, #4 and #5 dominate in North America (#4 and #5 are collectively known as Type 12). In Central and South America, there is no clear dominance of any genotype even though a few have relatively higher frequencies. Statistical analysis indicates significant differences among populations in Africa, Asia, Europe, North America, and Central and South America, with only Europe and North America exhibiting similar diversity. Collectively, the results revealed distinct population structures and geographical patterns of diversity in *T. gondii*.

Key words: *Toxoplasma gondii*, PCR-RFLP, genotyping, population structure, genetic diversity.

INTRODUCTION

Toxoplasma gondii is a parasitic protozoan parasite belonging to the phylum Apicomplexa. It parasitizes mammals and birds, and one third of the world's human population is infected (Dubey, 2010). *Toxoplasma gondii* generally forms chronic infections in its hosts, embedding in muscle and brain tissue (Tenter *et al*. 2000). In immunocompromised hosts such as AIDS patients, infection with *T. gondii* may result in encephalitis, and in developing fetuses acute toxoplasmosis may result in blindness, deformation, mental retardation or even death (Montoya and Liesenfeld, 2004). In veterinary medicine, toxoplasmosis is a major contributor to abortion in a number of economically important livestock animals such as sheep and goats (Buxton *et al*. 2007). Recently, chronic toxoplasmosis has been shown to cause behavioural changes in mice, making them attracted rather than repelled by the scent of cat urine, attributable to inhibition of neuronal function and alteration of neurotransmitter levels (Gatkowska *et al*. 2012; Haroon *et al*. 2012). In humans, the condition has also been shown to correlate with schizophrenia (Yolken *et al*. 2001; Torrey *et al*. 2007) and bipolar disorder (Pearce *et al*. 2012; Hamdani *et al*. 2013).

Unlike many other parasites in the phylum Apicomplexa, which exhibit stronger host specificity, *T. gondii* possesses an extremely broad host range, infecting both mammals and birds (Dubey, 2010). The parasite has been isolated from a wide range of hosts, including both terrestrial and marine animals. In addition to having this expansive host range, *T. gondii* also seems to be nearly ubiquitous geographically, having been isolated from a variety of climatic regions on every continent surveyed (Dubey, 2010). It seems that this expansion of the parasite occurred relatively recently in its evolutionary history (Khan *et al*. 2007). Because of the remarkable evolutionary success of *T. gondii* that has resulted in such broad expansion, it is important to understand transmission patterns and the factors that influence such patterns. In 1995, Howe and Sibley published a landmark article showing

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evidence for a clonal population structure in Europe and North America, in which the vast majority of *T. gondii* strains could be grouped into three lineages, namely Types I, II and III (Howe and Sibley, 1995). There appeared to be very limited sexual recombination between strains belonging to each of the lineages. While this model of the *T. gondii* population structure is still upheld to a large extent, it later became clear that the actual structure was more complex than was originally thought. This was found to be particularly true in Central and South America, where an abundance of atypical (non-clonal) strain types have been found (Ferreira *et al*. 2006; Lehmann *et al*. 2006; Khan *et al*. 2007; Pena *et al*. 2008; Su *et al*. 2012). Moreover, improvements in genotyping techniques have revealed greater complexity not only in Central and South America, but also in North America (Rajendran *et al*. 2012; Su *et al*. 2012).

Several molecular techniques including polymerase chain reaction – restriction fragment length polymorphism analysis (PCR-RFLP) (Howe and Sibley, 1995; Su *et al*. 2006, 2010), microsatellite DNA analysis (Ajzenberg *et al*. 2002, 2010) and multilocus DNA sequence typing of introns (Khan *et al*. 2007, 2011) have been used to study the genetic makeup of *T. gondii* strains. A comprehensive review of these methods and their contribution to the state of molecular epidemiology and population genetics of *T. gondii* can be found in a recent publication (Dardé *et al*. 2014). Among these methods, PCR-RFLP analysis of 10 markers (Su *et al*. 2006, 2010) was applied to more than 1000 *T. gondii* isolates worldwide, generating a significant amount of data and revealing the genetic diversity of the parasite. Here, we summarize the data generated to the end of 2012 using this approach. These data come from numerous publications originating from studies performed on every continent except Antarctica, and provide information for 1457 isolates representing 189 different genotypes. A number of *T. gondii* isolates previously typed by other methods were genotyped by the 10 PCR-RFLP markers in our laboratory and the results were integrated into this review article. Together, this information makes up the most comprehensive and cohesive picture of the global *T. gondii* population structure yet compiled.

GEOGRAPHICAL PATTERNS OF GENETIC DIVERSITY

At present, there is no standard nomenclature in designating genotypes for *T. gondii*. Conventional designation defined the clonal Type I, II and III lineages, and lumped together all others as atypical or exotic genotypes (Howe and Sibley, 1995; Grigg *et al*. 2001; Su *et al*. 2003). The subsequently identified major genotypes were added to the list including Type BrI, BrII, BrIII and BrIV, Type 12, Africa 1 and Chinese 1 (Pena *et al*. 2008; Mercier *et al*. 2010; Chen *et al*. 2011; Khan *et al*. 2011). However, this scheme of genotype designation is too cumbersome to define the hundreds of genotypes identified by the multilocus PCR-RFLP method. To overcome this problem, a scheme has been adopted in which each genotype is designated as a 'ToxoDB PCR-RFLP genotype' followed by a specific numeral. A comparison of conventional nomenclature and ToxoDB PCR-RFLP designations for major genotypes is provided in Table 1. To find a more comprehensive comparison of genotype designation schemes, see the recent publication by Dardé *et al*. (2014).

A total of 189 ToxoDB PCR-RFLP genotypes identified from 1457 samples are summarized in Table S1 – in Online version only. The compiled data were based on published results reported up to the end of 2012. The vast majority of *T. gondii* isolates were obtained from domestic and wild animals with chronic infection. For these samples, infection of *T. gondii* was first determined by the presence of anti-*Toxoplasma* antibodies in their sera, followed by bioassay of seropositive animal tissues (brain and/or muscle) in mice or cats. *Toxoplasma gondii* isolates obtained from experimentally infected mice or cats were further processed for genotyping. The global distribution of the 189 genotypes is illustrated in Fig. 1. The 10 most frequently identified are genotypes #2, #3, #1, #5, #4, #9, #6, #7, #8 and #10, accounting for 13·8, 12·6, 12·2, 5·0, 4·5 3·8, 3·3, 2·6, 2·3 and 2·1% of the samples, respectively. Genotypes #1 and #3, which differ only at the Apico locus, together compose the conventional Type II lineage and accounted for 24·8% (362/1457) of the population. Genotype #1 is also referred to as Type II clonal, whereas #3 as Type II variant (Table 1). As noted, genotype #2, also known as Type III, accounted for 13·8% (201/1457) of the samples. Genotypes #4 and #5, which differ only at the SAG1 locus and are collectively known as Type 12, accounted for 9·5% (139/1457) of the population. The results showed that genotype #1, #2 and #3 (Type II clonal, Type III and Type II variant) are identified worldwide. These three genotypes are highly prevalent in Europe. Genotypes #1, #2, #3, #4 and #5 dominate in North America. Genotypes #2 and #3 (Types III and II variant) dominate in Africa, and genotypes #9 and #10 (Chinese 1 and Type I) are prevalent in East Asia. In the paragraphs below, we have summarized the genotyping results by major geographical regions. Due to small sample size (only 3 isolates), Australia is not discussed further.

North America

In North America, including the USA and Canada, a total of 501 isolates (including 2 samples from Hawaii) have been typed to 40 genotypes. The majority of the samples (86·4%, 433/501) fall into genotypes #1, #2, #3, #4 and #5 (Fig. 1). Genotypes

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Table 1. *Toxoplasma gondii* genotype designations for common lineages

Conventional genotype designations	ToxoDB PCR-RFLP Genotypes	Representative isolates	References
Type I, type 1	#10	GT1	Su *et al*. (2012)
Type II, type 2 (type 2 clonal)	#1	PTG	Su *et al*. (2012)
Type II, type 2 (type 2 variant)	#3	PRU	Su *et al*. (2012)
Type III, type 3	#2	VEG	Su *et al*. (2012)
Type 12, atypical, exotic	#4	B41	Khan *et al*. (2011); Su *et al*. (2012)
Type 12, atypical, exotic, includes Type X and Type A	#5	ARI	Khan *et al*. (2011); Su *et al*. (2012)
Type BrI, atypical, exotic, Africa 1	#6	FOU, TgCatBr2	Pena *et al*. (2008); Mercier *et al*. (2010); Su *et al*. (2012)
Type BrII, atypical, exotic	#11	TgCatBr1	Pena *et al*. (2008); Su *et al*. (2012)
Type BrIII, atypical, exotic	#8	P89 (TgPgUs15), TgCatBr3	Pena *et al*. (2008); Su *et al*. (2012)
Type BrIV, atypical, exotic	#17	MAS, TgCkBr147	Pena *et al*. (2008); Su *et al*. (2012)
Chinese 1, atypical, exotic	#9	TgCtPRC4	Dubey *et al*. (2007*b*); Chen *et al*. (2011); Su *et al*. (2012)

#1 (29·7%, 149/501) and #3 (14·2%, 71/501), which are the conventional Type II lineage, accounted for 43·9% (220/501) of the population. Genotype #2 (Type III) accounted for 18·2% (91/501) of the samples. Genotypes #4 (10·0%, 50/501) and #5 (14·4%, 72/501) (together as Type 12) accounted for 24·4% (122/501) of the population. Type 12 is now recognized as the fourth clonal lineage in North America (Khan *et al*. 2011). It was shown that Type 12 is a dominant lineage in wildlife in North America (Dubey *et al*. 2011*b*). *Toxoplasma gondii* strains belonging to this lineage, also designated as Type X, were associated with high mortality in sea otters along the coast of California (Miller *et al*. 2008). Genotype #10 (Type I), previously considered as one of several dominant types in North America, accounted only for 0·8% (4/501) of the population.

Europe

Of the 64 samples obtained from European countries, 9 genotypes were identified (Fig. 1). Nearly three-quarters (64·1%, 41/64) belong to the genotypes #1 (25·0%, 16/64) and #3 (39·1%, 25/64), which are recognized as the Type II lineage. Genotype #2 (Type III) makes up 12·5% (8/64) of the samples. Genotype #10 (Type I) and genotype #6 (also known as Type BrI, Africa 1) both have relatively high frequencies, accounting for 9·4% (6/64) and 4·7% (3/64), respectively. This genetic uniformity gives Europe the closest resemblance to the three-lineage model proposed by Howe and Sibley (1995). The prevalence of genotypes #4 and #5 (also collectively designated as Type 12) in North America provides contrast with Europe, where genotype #4 has not been found and genotype #5 makes up only a minor part of the population. More sampling from a greater diversity of hosts is needed from European countries for better understanding of the region's *T. gondii* population structure.

Asia

In Asia, there also appears to be a high degree of genetic uniformity. From 102 samples, 10 genotypes were identified. Genotype #9 (Chinese 1) is by far the most commonly found, accounting for 48·0% (49/102) of samples. It is present in China, Vietnam and Sri Lanka, indicating a widespread distribution in Eastern Asia. Genotype #10 is also common in China, unlike in most other countries (13·7%, 14/102 in Asia). Genotypes #4, #18 and #20 have relatively high frequencies among the samples in Asia. Genotype #20 has been identified in a wide range of areas, from Sri Lanka in south Asia to Egypt in North Africa. Thus far, most of the sampling from Asia has been from the more populous eastern regions. More sampling is thus needed from the western areas where the climate and geography, as well as the distribution of human populations, are markedly different.

Africa

A total of 141 samples were typed and 13 genotypes identified in Africa. Most samples (118/141) in Africa were from Egypt. Overall, genotype #3 (45·4%, 64/141) and #2 (39·0%, 55/141) are the two dominant types, accounting for 84·4% of the samples. Genotype #6 was identified for several samples. The limited data that are available from sampling in Western Africa seem to suggest a high level of diversity, with a relatively low frequency of common genotypes. However, more sampling is required in Africa before strong conclusions can be drawn regarding the makeup of the *T. gondii* population of that continent. It would be of special interest to have more information regarding *T. gondii* in the tropical regions of Africa.

Central and South America

A total of 646 samples were typed and 156 genotypes identified in Central and South America. Unlike in

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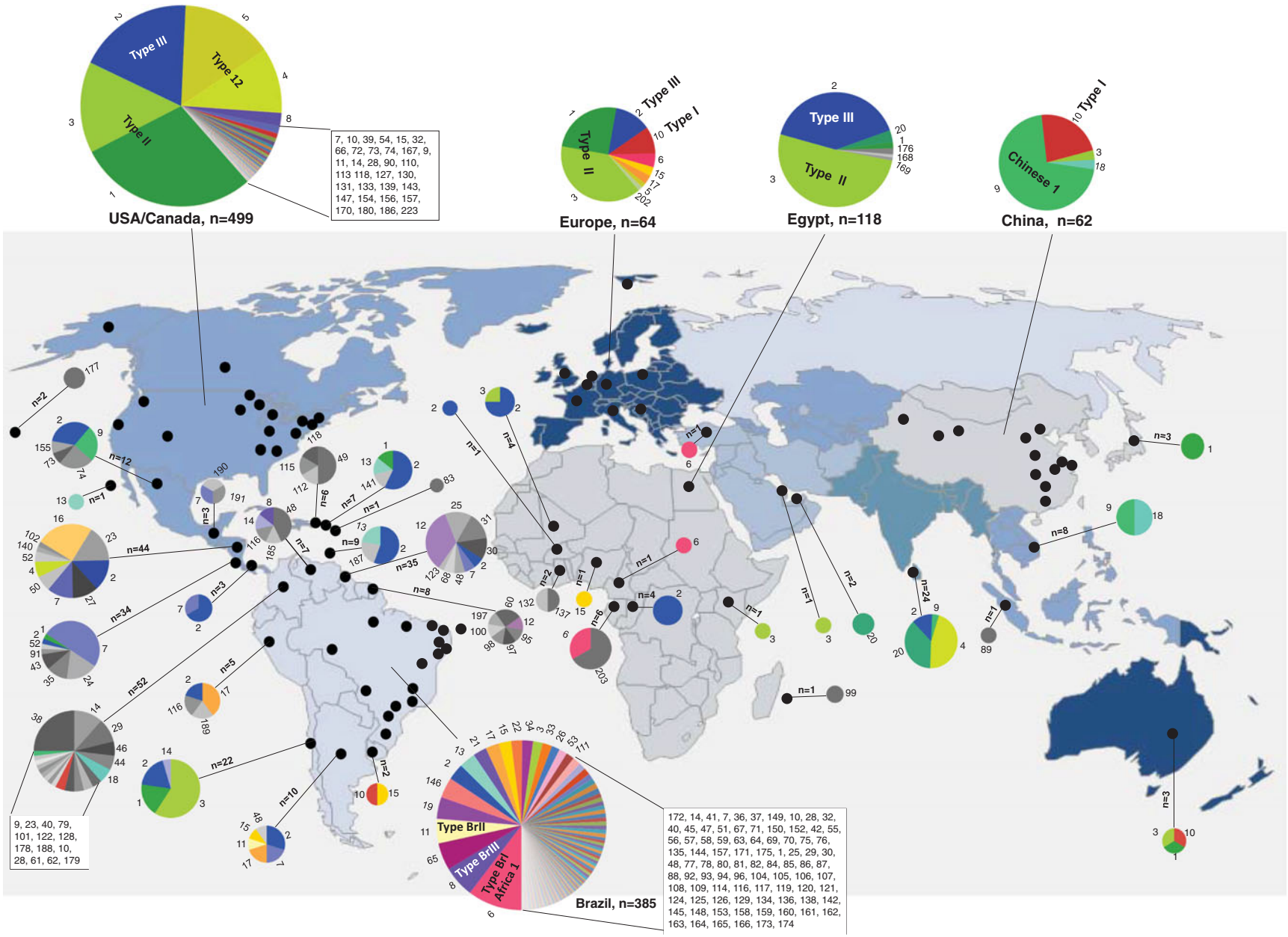

Fig. 1. Geographical distribution of *Toxoplasma gondii* genotypes. Black dots indicate locations from which *T. gondii* isolates were obtained and genotyped using the PCR-RFLP method. The numbers around pie chart edges indicate ToxoDB PCR-RFLP genotypes. In some cases, alternative strain type designations are overlaid on pie charts in bold lettering (also see Table 1 for correlations between nomenclatures). Sizes of pie charts correlate with total number of isolates (n), and colours indicate different genotypes. Two strains (GANGI and WIK) reported from Africa without specific countries of origin were listed in Supplemental Table S1, but not included on this map.

Table 2. Basic statistics of *Toxoplasma gondii* populations from different geographical regions

	Africa	Asia	Europe	North America	Central/South America
Number of isolates	141	102	64	501	646
Number of genotypes	13	10	9	40	156
Gene diversity	0·6433 ± 0·0256	0·7280 ± 0·0391	0·7679 ± 0·0351	0·8285 ± 0·0086	0·9792 ± 0·0017
Mean no. of pairwise differences over loci	5·5687 ± 2·6895	4·9293 ± 2·4182	5·4365 ± 2·6495	5·0968 ± 2·4762	6·1121 ± 2·9118

Due to small sample size of only 3 isolates, Australia was not included for analysis.

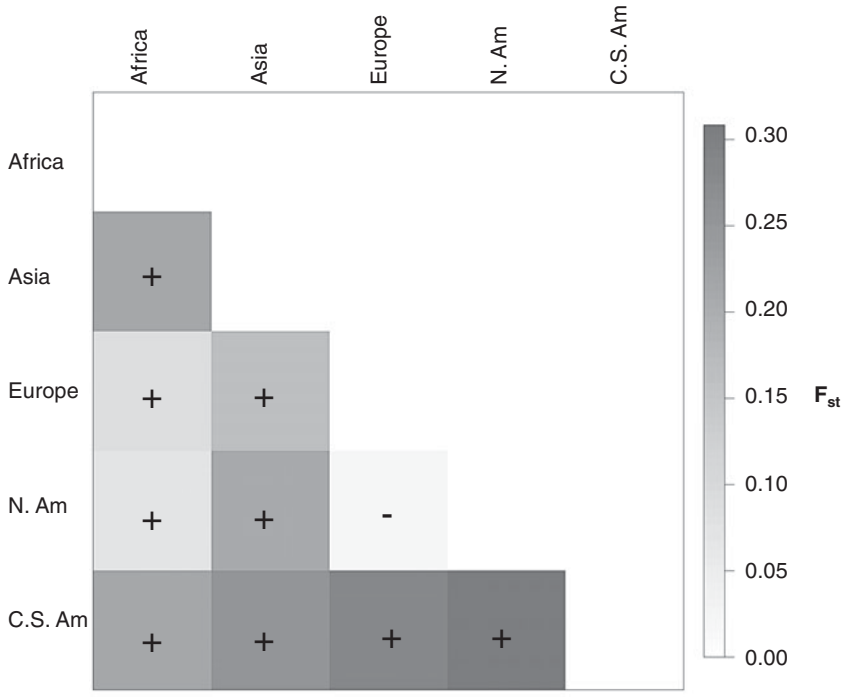

Fig. 2. Pairwise Fst of five *T. gondii* populations from different geographical regions. Comparison of populations was conducted using Arlequin ver 3.5. Statistical significance is determined at $P = 0{\cdot}001$. The '+' sign indicates significant difference between two populations, whereas '−' indicates non-significance. N. Am = North America, C. S. Am = Central and South America. The heat map indicates the Fst value. Due to small sample size (only 3 isolates), Australia was not included for analysis.

the northern hemispheric countries discussed above, *T. gondii* in this region displays a highly diverse population structure, in which no genotype appears to be clearly dominant. The top 10 genotypes are #2, #6, #7, #8, #11, #3, #65, #13, #19 and #146, with frequencies of 6·8, 6·2, 5·0, 3·7, 3·3, 3·1, 3·1, 2·5, 2·5 and 2·3%, respectively. The genotypes #1, #2, #3 and #10 are at low frequencies and have not taken over the genetic landscape as in the north. Of these genotypes, the #2 (Type III) is the most frequently occurring (6·8%, 44/646) and is found in many of the countries in this region, even those for which the sample size is small, such as Panama, Peru and Grenada. Even in these regions however, genotype #2 is often less frequent than many other genotypes. An exception to this trend is found in Chile, where we see a genetic makeup more similar to that found in North America and Europe, in which genotypes #1, #2 and #3 predominate. Chile is located on the west side of the Andes Mountains which separate it from most of the countries in South America. On its west side is the Pacific Ocean, which provides ports for maritime transportation to other continents. Trading with countries on different continents might bring genotypes #1, #2 and #3 to Chile, eventually allowing them to become dominant in this region (Rajendran *et al*. 2012).

Quantitative comparison of genetic diversity

To quantify differences of genetic diversity among *T. gondii* populations from major geographical areas, we analysed the typing data based on different continents. Within and between population diversity, and shared genotypes were analysed by Arlequin v3.5 as described in previous reports (Excoffier and

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Table 3. Shared genotypes among geographical regions

	Africa	Asia	Europe	North America
Africa				
Asia	1, 2, 3, 6, 20			
Europe	1, 2, 3, 6, 15	1, 2, 3, 6, 10		
North America	1, 2, 3, 15	1, 2, 3, 4, 9, 10	1, 2, 3, 5, 10, 15	
Central/South America	1, 2, 3, 6, 15	1, 2, 3, 4, 6, 9, 10, 18	1, 2, 3, 6, 10, 17	1, 2, 3, 4, 7, 8, 9, 10, 11, 14, 15, 28, 32, 73, 74, 118, 157

Due to small sample size of only 3 isolates, Australia was not included for analysis in this table.

Lischer, 2010; Rajendran *et al*. 2012). The results are summarized in Table 2, Fig. 2 and Table 3. Among the five major regions including Africa, Asia, Europe, North America and Central/South America, the genotype diversity (gene diversity) indices are 0·6433, 0·7280, 0·7679, 0·8285 and 0·9792, respectively, indicating high diversity in Central/South America, followed by North America (Table 2). Pairwise comparison revealed that there are significant differences among populations in Africa, Asia, Europe, North America and Central/South America, with only the populations of Europe and North America exhibiting similar genetic composition to each other (Fig. 2). Furthermore, the difference between Central/South America and other areas is more prominent (Fig. 2). Analysis of shared genotypes revealed the distribution of common genotypes (Table 3). Genotypes #1, #2 and #3 exist in all five continents. Genotype #6 (BrI, Africa 1) has been identified in all but North America, genotype #10 (Type I) has been identified in all but Africa, and genotype #15 identified in all but Asia. The North and Central/South Americas shared 17 genotypes, the highest among all populations compared.

DISCUSSION

The compiled PCR-RFLP typing results of 1457 *T. gondii* samples revealed 189 genotypes. Overall, geographical differences are readily recognized. Only a few genotypes dominate in the northern hemisphere, whereas hundreds of genotypes coexist with a few having a relatively higher frequency in the southern hemisphere. These results suggest a clonal population structure in the north and an epidemic population structure in the south. This finding is in strong agreement with the conclusion from a smaller sample size that was analysed recently (Dardé *et al*. 2014). Though the frequencies of genotypes may change with more intensive sampling, we expect that the compiled results of 1457 samples have captured the big picture of global *T. gondii* diversity.

Understanding *T. gondii* population structure is of great interest, as it may provide us with essential information regarding the transmission and evolution of this widespread zoonotic parasite. Several major factors may have been involved in shaping the modern-day *T. gondii* population structure. First, the rise of human agriculture about 11 000 years ago in Mesopotamia and its expansion to the New World in the last few hundred years may have significantly transformed our biological environment and reduced genetic diversity of animal parasites (Rosenthal, 2009). In North America, Europe and East Asia, large-scale farming and the erection of cities have largely transformed the landscape throughout much of these regions, and likewise many of the ancestral wild animal species have been replaced with genetically uniform domestic animals. Such transformation of geography and reduced animal diversity may have led to expansion of a few *T. gondii* genotypes that adapted to the new environment. The limited data lend support to the idea that the clonal *T. gondii* lineages may have become dominant in a manner that was concomitant with the beginnings of human agriculture in the region of Mesopotamia, whereupon these lineages then spread outward alongside the flow of human migration. However, more data are certainly required from the Middle East and other regions before more definitive conclusions can be drawn regarding this matter.

Second, the low genetic diversity of *T. gondii* observed in the northern hemisphere may be a result of the founder effect. The high genetic diversity observed in South and Central America suggests that *T. gondii* originated in this region (Lehmann *et al*. 2006). It is possible that a small number of founding strains were introduced from South America to other continents by maritime trading of goods, transportation of pet animals such as cats, or accidental transport of infected rodents within the last five centuries (Lehmann *et al*. 2006). These founder populations could then have expanded throughout their respective geographical regions with little competition or recombination between strains, leading to the observed clonal population structures.

Another possible explanation for the high genetic diversity found in South and Central America, in contrast to the low diversity of the northern hemisphere, is that the tropical and subtropical zones in the former region support a greater diversity and number of animal hosts, each of which might

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favour the selection of different *T. gondii* genotypes, enabling a wider variety of strains to proliferate. Additionally, the warmer climate may allow a greater number of oocysts to survive in nature for a longer period of time, resulting in more infection of hosts overall as well as decreased selective pressure compared with the northern hemisphere. Thus far, sampling in tropical regions other than those of South and Central America has been sparse and so understanding of the *T. gondii* population structures in these areas remains limited. If additional sampling were to reveal that regions with similar climate and host species diversity such as those found in Africa and southeast Asia also supported highly diverse *T. gondii* populations, such a finding would lend credence to this alternative hypothesis.

FUTURE PERSPECTIVE

The existing genotyping studies have revealed essential information regarding the global diversity of *T. gondii* and its population structure, providing us with preliminary data for further study on the origin, transmission, ecology and evolution of this highly successful protozoan parasite. To start tackling these topics, more in-depth sampling of *T. gondii* is necessary for several aspects. First, sampling of *T. gondii* in wildlife from remote areas that are not disturbed by human civilization is needed to better assess the effect of human settlement on the diversity of *T. gondii*. A recent study in French Guiana showed that a human environment may favour clonal expansion and reduce *T. gondii* diversity (Mercier *et al*. 2011). Given that human agriculture arose in the Fertile Crescent about 11 000 years ago in Mesopotamia (Diamond and Bellwood, 2003), it might have led to expansion of genotypes #1, #2 and #3 and significantly reduced *T. gondii* diversity in Europe and Southwest Asia. However, we would expect that high genetic diversity may still be maintained in wildlife from remote geographical regions that have not been disturbed by human settlement in Europe and Southwest Asia. Similar studies are also necessary in tropical Africa, the Amazon rain forest, South Asia and Australia to better understand the effect of human societies on *T. gondii* genetic diversity. Availability of these data will allow us to understand the global transmission patterns of *T. gondii*.

Second, potential long-distance transmission of *T. gondii* by migratory marine mammals and birds is poorly understood. It was shown that striped and bottle-nosed dolphins, as well as a variety of Antarctic pinnipeds, were seropositive for *T. gondii* infection (Dubey *et al*. 2007*a*, 2008; Rengifo-Herrera *et al*. 2012). Dolphins may migrate over relatively large distances and thus may be important vectors for the long-distance spread of *T. gondii*. Several studies have focused on the prevalence of *T. gondii* in marine mammals such as sea otters along the coastlines of California, Washington and Alaska. A genotype designated as 'Type X' (genotype #5) was dominant in these sea otters (Conrad *et al*. 2005; Sundar *et al*. 2008). The same genotype seemed to be the major cause of high mortality in otters in California (Conrad *et al*. 2005). Though sea otters are not migratory themselves, they may be sentinels of a route by which *T. gondii* might be introduced into the marine ecosystem (or at least an indicator of the types of strains present in such ecosystems), through which it seems likely that intercontinental transmission may occur. Migratory birds may also be an important contributor to the transmission of *T. gondii* between continents. However, few studies have been performed targeting such species. A recent study reported genotyping of a single isolate from a wild pigeon in Mexico (Alvarado-Esquivel *et al*. 2011). Other studies have found sporadic cases of *T. gondii* infection in wild bird species (Dubey *et al*. 2011*a*, *b*). Given the potential role of migrating marine mammals and birds in long-distance transmission of *T. gondii*, more studies on these animals are needed to better understand their contribution to the evolution of *T. gondii* on a global scale.

Third, most *T. gondii* samples studied so far have been from non-human animal origin. Only a limited number of samples have been isolated from human infections. A total of only 84 human samples have been analysed by the 10 PCR-RFLP markers and included in this review article. These samples have revealed a variety of genotypes with no clear pattern emerging. Atypical genotype #65 was found to occur in human patients in Sao Paulo, Brazil in a single study (Ferreira *et al*. 2011). Another feature of note seems to be that genotype #10 (Type I), the highly mouse-virulent lineage, appears to be more prevalent in human infections than is seen in other hosts. A larger sample size is necessary to determine the significance of this feature, and human toxoplasmosis patients need to be sampled from a greater variety of geographical regions in order to determine whether this is a global phenomenon. It would also be of interest to determine the mouse-virulence of the atypical strains found in human hosts in order to investigate the possibility that such murine virulence also leads to an increased ability to cause disease in *Homo sapiens*.

SUPPLEMENTARY MATERIAL

To view supplementary material for this article, please visit http://dx.doi.org/S0031182013001844

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