

# Genetic Diversity of *Toxoplasma gondii* Strains from Different Hosts and Geographical Regions by Sequence Analysis of *GRA20* Gene

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**Abstract:** *Toxoplasma gondii* is a eukaryotic parasite of the phylum Apicomplexa, which infects all warm-blood animals, including humans. In the present study, we examined sequence variation in dense granule 20 (*GRA20*) genes among *T. gondii* isolates collected from different hosts and geographical regions worldwide. The complete *GRA20* genes were amplified from 16 *T. gondii* isolates using PCR, sequence were analyzed, and phylogenetic reconstruction was analyzed by maximum parsimony (MP) and maximum likelihood (ML) methods. The results showed that the complete *GRA20* gene sequence was 1,586 bp in length among all the isolates used in this study, and the sequence variations in nucleotides were 0-7.9% among all strains. However, removing the type III strains (CTG, VEG), the sequence variations became very low, only 0-0.7%. These results indicated that the *GRA20* sequence in type III was more divergence. Phylogenetic analysis of *GRA20* sequences using MP and ML methods can differentiate 2 major clonal lineage types (type I and type III) into their respective clusters, indicating the *GRA20* gene may represent a novel genetic marker for intraspecific phylogenetic analyses of *T. gondii*.

**Key words:** *Toxoplasma gondii*, sequence variation, dense granule 20 (*GRA20*), phylogenetic analysis

*Toxoplasma gondii*, one of the most successful intracellular protozoan parasites, can infect the majority of vertebrate species including humans with a worldwide distribution [1-3], and approximately one-third of the population has been exposed to *T. gondii*. Normally, the infections are asymptomatic or sub-clinical. However, the *T. gondii* infection can cause abortion and stillbirth in pregnant women, and encephalitis, chorioretinitis, and systemic infections in immunocompromised individuals [2]. In animals, *T. gondii* can also cause abortion in livestock, especially in sheep and goats, which can spawn a great number of economic losses in livestock [3]. However, there was no effective vaccine and drugs that can help to control toxoplasmosis.

The strains of *T. gondii* that predominate in Europe and North America, classified into types I, II, and III, differ in a

wide range of phenotypes, including virulence, persistence, migratory capacity, and how they interface with the immune response [4-6]. Thus, the information of genetic diversity of *T. gondii* is useful for better understanding epidemiological patterns and pathogenicity, as well as exploring of new polymorphic virulence effectors.

*GRA20*, a novel dense granule protein, is secreted and targeted to parasitophorous vacuole membrane (PVM), which may participate in the manipulation of the host immunity [7]. Previous studies have identified the existence of polymorphisms in dense granule proteins, such as *GRA15*, *GRA5*, and *GRA6* [8-10], but the sequence variation about the *GRA20* gene among different *T. gondii* isolates is still unknown. Therefore, the objective of this study was to examine sequence diversity of *GRA20* gene among *T. gondii* strains from different hosts and geographical regions worldwide.

In this study, a total 16 *T. gondii* strains from different hosts and geographic locations were used for analysis (Table 1). These *T. gondii* isolates have been genotyped and genomic DNA (gDNA) was prepared as described previously [11-13].

To acquire amplicons of *GRA20* genes concerning different *T.*

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**Table 1.** Details of *Toxoplasma gondii* isolates used in this research

Isolate	Host	Geographical location	Genotype <sup>a</sup>
RH	Human	France	Reference, type I, ToxoDB #10
GT1	Goat	United States	Reference, type I, ToxoDB#10
SH	Human	Shanghai, China	type I, ToxoDB #10
TgCatBr9	Cat	Brazil	ToxoDB#42
VEG	Human	United States	Reference, ToxoDB#2
ME49	Sheep	United States	type II, ToxoDB#1
TgCatBr64	Cat	Brazil	Reference, ToxoDB#111
TgCatBr5	Cat	Brazil	Reference, ToxoDB#19
PRU	Human	France	type II, ToxoDB #1
QHO	Sheep	Qinghai, China	type II, ToxoDB #1
PTG	Sheep	United States	Reference, type II, ToxoDB#1
TgC7	Cat	Guangzhou, China	ToxoDB #9
PYS	Pig	Panyu, China	ToxoDB #9
CTG	Cat	United States	Reference, type III, ToxoDB#2
TgWtdSc40	Deer	USA	type 12, ToxoDB#5
TgToucan	Toucan	Costa Rica	Reference, ToxoDB#52

<sup>a</sup>Based on the results of Zhou et al. [11,12] and Su et al. [13].

**Table 2.** Characteristics of *Toxoplasma gondii* GRA20 (*TgGRA20*) gene sequences

Item	DNA		CDS <sup>a</sup>		First Extron		First Intron		Second Extron	
	ALL <sup>b</sup>	Except III <sup>c</sup>	ALL	Except III	ALL	Except III	ALL	Except III	ALL	Except III
Length (bp)	1,586	1,586	1,242	1,242	140	140	344	344	1,102	1,102
T+A (%)	44.96-45.40	45.02-45.40	42.83-43.32	42.91-43.32	45.71-46.43	45.71-46.43	52.62-52.91	52.62-52.91	42.47-42.92	42.56-42.92
Transition	59	12	57	10	1	1	2	2	56	9
A↔G	33	7	31	5	1	1	2	2	30	4
C↔T	26	5	26	5	/	/	/	/	26	5
Transversion	62	3	61	2	0	0	1	1	61	2
A↔T	8	/	8	/	/	/	/	/	8	/
G↔C	17	1	17	1	/	/	/	/	17	1
A↔C	19	2	18	1	/	/	1	1	18	1
G↔T	18	/	18	/	/	/	/	/	18	/
loss	6	0	6	0	0	0	0	0	6	0
VN <sup>d</sup>	127	15	124	12	1	1	3	3	123	11
R <sup>e</sup>	0.95	4	0.93	5	\	\	2	2	0.92	4.5
Distance (%)	0-7.9	0-0.7	0-10.1	0-0.6	0-0.7	0-0.7	0-0.9	0-0.9	0-11.4	0-0.6

<sup>a</sup>CDS: coding sequence.

<sup>b</sup>All: all the *T. gondii* in this study.

<sup>c</sup>Except III: all the *T. gondii* except CTG and VEG.

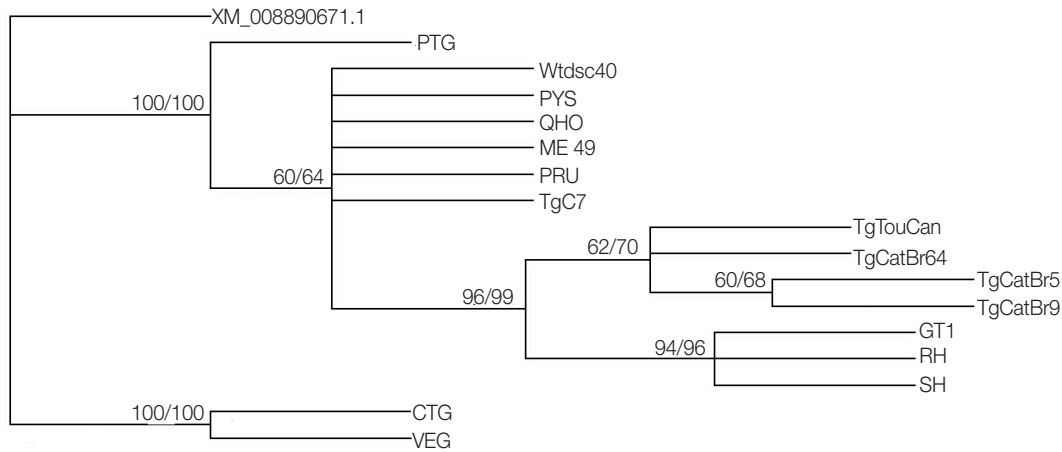
<sup>d</sup>VN: variable nucleotide.

<sup>e</sup>R = transition/transversion.

*gondii* isolates, the primers GRA20-F (5'- ATGCATAGCCG-GAACTGCGTC-3') and GRA20-R (5'- TCACGCGGGCTTTC-TACGG-3') were designed based on *T. gondii* ME49 strain available in ToxoDB database (TGME49\_200010). All the PCR products of GRA20 genes were purified by the DNA purification kit (GenStar, Beijing, China), ligated into pMD18-T vector (TaKaRa, Dalian, China), and then transformed into JM109 competent cells (Promega, Madison, Wisconsin, USA). Subse-

quently, the positive colonies were screened by PCR, and then sequenced by GenScript Co., Ltd. (Nanjing, China).

The acquired *GRA20* gene sequences were aligned by the Multiple Sequence Alignment Program, Clustal X 1.83 [14], and sequence variation was determined among the examined *T. gondii* strains. Phylogenetic reconstructions based on the complete sequences of *GRA20* gene among 13 *T. gondii* isolates and plus the corresponding sequences of strains TgCatBr9,



**Fig. 1.** Phylogram of 16 *Toxoplasma gondii* isolates determined by analysis of the entire sequences of the *GRA20* gene. The tree was reconstructed by maximum parsimony (MP) and maximum likelihood (ML) analyses. The numbers along branches indicate bootstrap values resulting from different analyses in the order: MP/ML.

VEG, and ME49 available in ToxoDB (<http://toxodb.org/toxo/>) were carried out by 2 inference methods, maximum likelihood (ML) and maximum parsimony (MP) methods by Paup, with the sequence of *Hammondia hammondi* (XM\_008890671.1) as the out-group. Phylograms were drawn by the Tree View program version 1.65.

In the present study, the obtained entire genomic sequences of the *GRA20* gene were 1,586 bp in length in all examined isolates. According to the analysis of all the 16 *GRA20* complete sequences, there were 2 exons and 1 intron in the *GRA20* gene (Table 2). The A+T content ranged from 45.0% to 45.4% in the entire sequence. There were 124 nucleotide position variations with a distribution of 57 transitions (A↔G and C↔T), 61 transversions (C↔G, T↔G, A↔C, and A↔T) in CDS, and 2 transitions (A↔G and C↔T), and 1 transversion (C↔G, T↔G, A↔C, and A↔T) in the intron (Table 2). However, when we analyzed the *GRA20* sequences without type III (CTG, VEG) strains, there were 12 nucleotide variations with a distribution of 10 transitions (A↔G and C↔T), 2 transversions (C↔G, T↔G, A↔C, and A↔T) in CDS, and 2 transitions (A↔G and C↔T) and 1 transversion (C↔G, T↔G, A↔C, and A↔T) in the intron. The alignment of *GRA20* gene sequences showed that sequence variation was 0-7.9% in all studied strains, while the sequence variation became 0-0.7% without the CTG and VEG strains. Phylogenetic reconstruction of all 16 *T. gondii* strains based on *GRA20* sequence data showed that the type I and type III of *T. gondii* strains were clustered into respective clusters separately (Fig. 1).

Recently, polymorphisms in the sequences of *GRA5*, *GRA6*, *GRA7*, and *GRA15* genes have been reported [8,9,15,16]. Among them, polymorphic dense granule proteins were widely used in typing *T. gondii* isolates, such as *GRA6* [10]. Furthermore, polymorphic dense granule protein may have different roles in regulating the inflammatory response. For example, *GRA15* in type II activate more IL-12 than type I or type III strains [8]. In this study, we found *GRA20* gene was very diverse in type III, indicating the functions may be different, too. Our results were consistent with that of some previous studies using other genetic markers, such as *GRA5*, *Rop17*, and *HSP60* for genotyping [9,17,18], but different to some previous studies, such as *Rop38* and *eIF4A* [19,20].

In conclusion, the present study examined the sequences of the *T. gondii* *GRA20* gene and revealed that it was more divergence in type III compared to other *T. gondii* strains, suggesting the functions of *GRA20* in type III may be different from other strains. Phylogenetic analysis indicated that the *GRA20* gene could distinguish the type I and type III strains, suggesting the *GRA20* gene may be a novel genetic marker for studying genetic variation or the population genetic structures of *T. gondii* isolates.

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## CONFLICT OF INTEREST

The authors declare that they have no competing interests.

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