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Limited sequence variation in rhoptry protein 41 gene among *Toxoplasma gondii* isolates from different hosts and geographical locations

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Abstract. *Toxoplasma gondii* is a medically and agriculturally important protozoan parasite that can infect virtually all the mammalian and avian species. Previous studies showed that the family of rhoptry proteins (ROPs) plays a key role in the invasion process of *T. gondii*, and its several members can be potential marker for population genetic researches of *Toxoplasma*. In order to estimate whether other member is also suitable as the novel genetic marker, the variation of ROP41 gene among 11 *T. gondii* isolates from different hosts and geographical locations and two reference strains was examined in this study. Our results showed that all the examined sequence of *Tg*ROP41 gene was 1473 bp in length, and their A+T contents were between 48.47% and 48.88%. Sequence analysis presented 14 nucleotide mutation positions (0%-0.54%), leading to 5 amino acid substitutions (0%-0.61%) through alignment with *T. gondii* ME49 strain (ToxoDB: *TG*ME49_266100). Furthermore, phylogenetic analyses by MP and BI methods based on deduced amino acid sequences of *Tg*ROP41 gene was only able to distinguish the type I strain, but not able to separate the two classical genotypes (Type II and III) into the respective clusters. These results indicated limited sequence diversity in the *Tg*ROP41 gene.

INTRODUCTION

Toxoplasma gondii with worldwide distribution is an obligate intracellular parasite of medical and veterinary significance, which can infect any warm-blooded animals including humans (Tenter *et al.*, 2001; Dubey, 2009). *T. gondii* infection can cause severe diseases especially for immunocompromised patients and pregnant women, though common infection usually leads to asymptomatic or sub-clinical symptoms (Zhou *et al.*, 2011). Furthermore, this protozoan parasite can also result in abortion or congenital toxoplasmosis in livestock with considerable economic losses (Hunter *et al.*, 2012; Dubey *et al.*, 2005).

The rhoptries are secretory subcellular organelles of apicomplexan parasites that contain proteins play a key role in invasion and adjustment of the host cell (Reese *et al.*, 2012; Camejo *et al.*, 2014). Rhoptry proteins (ROPs) are released during invasion but they do not form methodic structures and are not found at the moving junction (MJ). Instead, following release, they migrate to one of three common locations: the entocoel of the nascent parasitophorous vacuole; the parasitophorous vacuole membrane; or the inner of the parasitifer cell (Ravindran *et al.*,

2008). Previous studies have demonstrated that many members of ROPs family can be used as vaccine candidates against *T. gondii* infection, such as ROP16 and ROP18 (Bhopale, 2003), and can be suitable markers for *T. gondii* population genetic studies (Dlugonska, 2008). Sequence variation in different ROP genes is usually low, eg. 1.7% in ROP7, 2.0% in ROP13 and 1.1% in ROP38 (Xu *et al.*, 2014). Therefore, a novel rhoptry protein with low sequence variation may be deemed to open up new vaccines against *T. gondii* infection.

Nevertheless, little is known about sequence variation in *Tg*ROP41 gene among *T. gondii* isolates of different genotypes. The aim of the present study was to inspect the sequence variation in *Tg*ROP41 genes among *T. gondii* isolates from different hosts and geographical regions, and to evaluate whether the *Tg*ROP41 gene sequence may represent a new marker for studying *T. gondii* population genetic structures.

MATERIALS AND METHODS

T. gondii isolates

A total of 11 *T. gondii* isolates originating from different hosts and geographical regions were used in present study (Table 1) (Su *et al.*, 2010), and genomic DNA (gDNA) of these *T. gondii* isolates was prepared and genotyped in our previous studies. Two reference strains (ME49: *TG*ME49_266100; VEG: *TG*VEG_266100) available in ToxoDB database were included for comparative analysis.

PCR amplification

In order to obtain the sequences of *Tg*ROP41 genes, gDNA of individual isolates was used as template for the amplification of the entire *Tg*ROP41 gene sequences. One pair of specific primers (forward primer: 5'-A*TG*CGTCACG*TG*TTCAACT-3'; reverse primer: 5'-TTAGGAAAGCACT*TGTG*AGGTC-3') was designed based on the reference sequence of *T. gondii* ME49 strain (ToxoDB: *TG*ME49_266100). PCR reactions were carried out in 25 µL containing 12.5 µl Premix Taq (TaKaRa, China), ROP41F (20 pmol) 0.5 µl, ROP41R (20 pmol) 0.5 µl, template DNA (200 ng) 2 µl, and ddH_2O 9.5 µl. The PCR reaction was carried out in a thermocycler (Bio-Rad) with an initial denaturation at 94ºC for 4 min, followed by 35 cycles of 94ºC for 30 sec (denaturation), 56ºC for 30 sec (annealing), 72ºC for 1.5 min (extension), and a final extension of 72ºC for 10 min. A negative control sample without gDNA was included in each PCR reaction. Each amplicon (6 µl) was examined on 1% (w/v) agarose gel to appraise amplification efficiency. Sizes of *Tg*ROP41 PCR products were approximated by using a DNA marker (DL2000, TAKARA),

Table 1. Details of *Toxoplasma gondii* isolates used in the present study

No.	Isolate	Host	Geographical Location	Genotype*
1	RH	Human	France	Reference, Type I, ToxoDB #10
2	GT1	Goat	United States	Reference, Type I, ToxoDB#10
3	PLH	Pig	Henan, China	Type I, ToxoDB #10
4	MAS	Human	France	Reference, ToxoDB#17
5	*Tg*CatBr5	Cat	Brazil	Reference, ToxoDB#19
6	PRU	Human	France	Type II, ToxoDB #1
7	QHO	Sheep	Qinghai, China	Type II, ToxoDB #1
8	*Tg*C7	Cat	Guangzhou, China	ToxoDB #9
9	PYS	Pig	Panyu, China	ToxoDB #9
10	GJS	Pig	Jingyuan, Gansu, China	ToxoDB #9
11	CTG	Cat	United States	Reference, Type III, ToxoDB#2

*Based on the results of Su *et al.* (2010).

and photographed using a gel documentation system (UVP GelDoc-It™ Imaging System, Cambridge, U.K.).

Sequencing of the *Tg*ROP41 amplicons

Positive *Tg*ROP41 amplicons were purified using the spin columns according to the manufacturer's recommendations (Wizard™ SV Gel and PCR Clean-Up System, Promega, USA), ligated with pMD18-T vector (TaKaRa, China), and then transformed into the Trans5α competent cells (TransGen, China). Following the screening by PCR amplification, and then the positive colonies were sequenced by Shanghai Songon Biological Engineering Biotechnology Company, China.

Sequence analysis and phylogenetic reconstruction

All the obtained *Tg*ROP41 sequences were aligned with Clustal X 2.11, and their evolutionary analysis was performed by MEGA 5.2 (Thompson *et al.*, 1997). The variation of intraspecific sequence was assessed by the percentage of different bases. Phylogenetic reconstructions of all the examined *T. gondii* isolates based on deduced amino acid sequences of the *Tg*ROP41 gene were carried out using two inference methods, namely maximum parsimony (MP) and Bayesian inference (BI). MP analysis was performed using PAUP* 4.0b4a (Swofford *et al.*, 2002) with indels treated as missing character states. A total of 1,000 random addition searches using TBR were run for each MP analysis. Bootstrap probability (BP) was calculated from 1,000 bootstrap replicates with 10 random additions per replicate in PAUP. BI analyses were conducted with four independent Markov chains run for 10,000,000 metropolis-coupled MCMC generations, sampling a tree every 10,000 generations in MrBayes 3.1.1 (Rodquist *et al.*, 2013). The first 250 trees were omitted as burn-ins and the remaining trees were used to calculate Bayesian posterior probabilities (PP). Phylograms were drawn using the Tree View program version 1.66 (Page *et al.*, 1996).

RESULTS AND DISCUSSION

Our results showed that the sequence of *Tg*ROP41 gene was 1473 bp in length for all of the examined strains, and their A+T contents varied from 48.47% to 48.88%. Sequence analysis presented 14 nucleotide mutation positions (0%-0.54%) including 13 transitions (C<–>T, T<–>G, A<–>C, and A<–>G) and 1 transversion (A<–>T) (Figure 1A), resulting in 5 amino acid substitutions (0%-0.61%) (Figure 1B) in contrast to that of the reference strain ME49 (ToxoDB: *TG*ME49_266100), which is lower than that of ROP7, ROP13 and ROP47 (Wang *et al.*, 2015). Three isolates (GT1, RH and PLH) representing the genotype I strain had the identical *Tg*ROP41 sequences, the three ToxoDB #9 strains (GJS, *Tg*C7 and PYS) also had identical sequences, while the two III strains (VEG and C*TG*) also had the same sequences. These results revealed that variation in *Tg*ROP41 sequences among the examined *T. gondii* isolates was really low, and similar results were also found in previous studies, such as PLP1, MIC13, HSP60 and other genes among the clonal lineages of *T. gondii* (Lu *et al.*, 2014).

Phylogenetic analyses by MP and BI methods based on deduced amino acid sequences of *Tg*ROP41 gene was shown in Figure 2. Our study indicated that it was able to distinguish the type I strain, but was not able to separate the examined two classical genotypes (Type II and III) and ToxoDB #9 into the respective clusters, and more *T. gondii* strains from different hosts and geographical locations were needed to test this possibility. This result is similar to previous studies that using GRA5 (Chen *et al.*, 2012) and ROP38 (Xu *et al.*, 2014) as genetic markers.

In summary, our data indicated the existence of low sequence variability in *Tg*ROP41 gene among the examined *T. gondii* isolates from different hosts and geographical locations, thus it is not a suitable marker for population genetic studies of *T. gondii* strains. Due to the very low sequence variability in *Tg*ROP41 gene

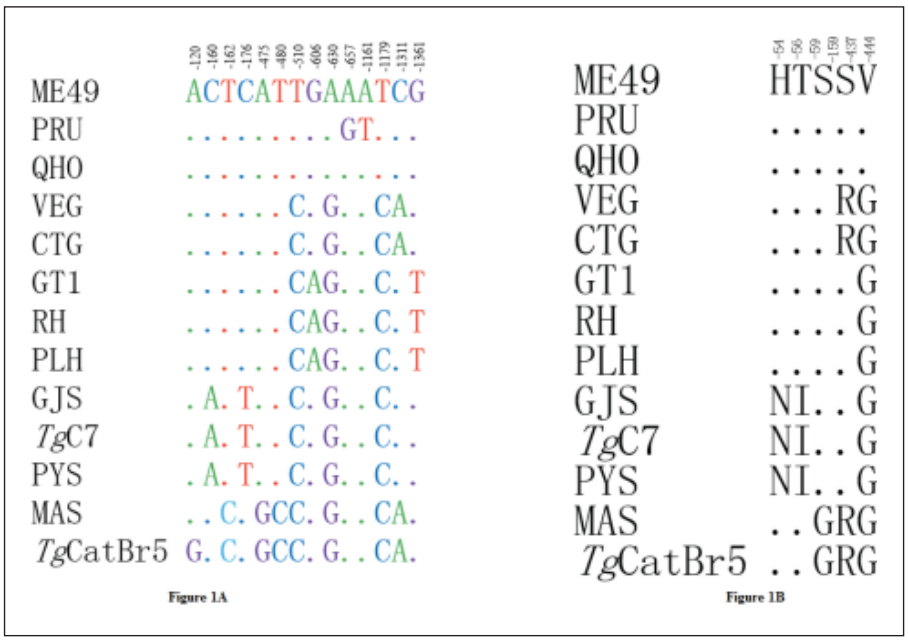

Figure 1. Multiple alignment analyses for nucleotides (A) and amino acid sequences (B) of *Toxoplasma gondii Tg*ROP41 gene. Point (.) stands for identical nucleotide or amino acid in comparison to that of *T. gondii* ME49 strain (upper line), and the numbers indicate the variable sequence positions for nucleotide (A) and amino acid (B).

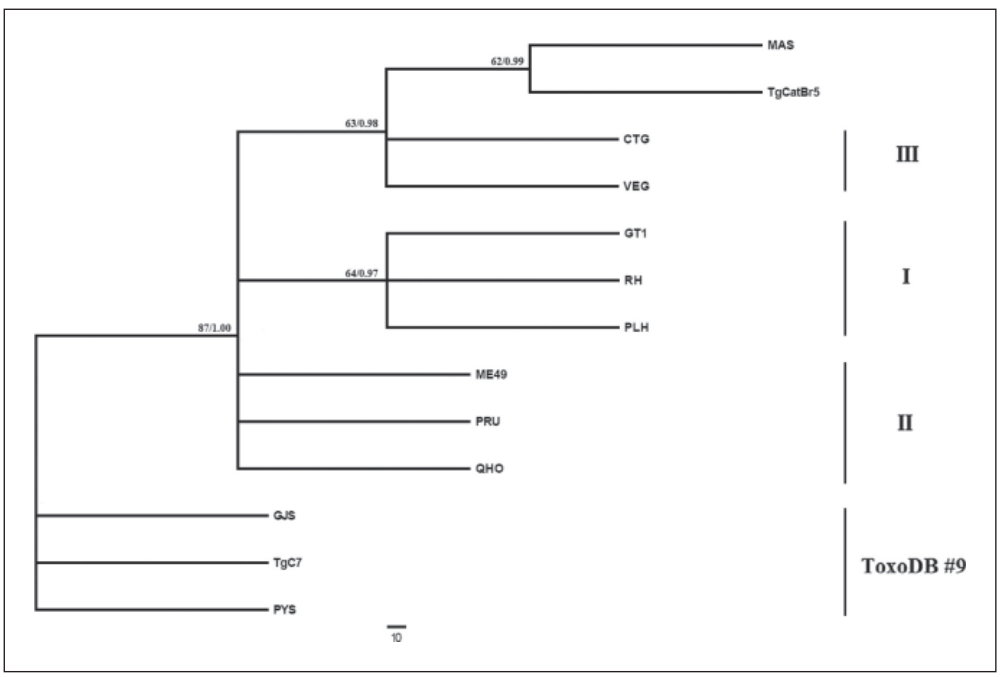

Figure 2. Phylogram of 13 *Toxoplasma gondii* isolates including two reference strains (ME49 and VEG) using maximum parsimony (MP) and Bayesian inference (BI) analyses. The numbers along the branches indicate bootstrap values in the order of MP and BI, and the strains belonging to three classical genotypes or ToxoDB #9 were denoted.

between different *T. gondii* isolates, it may represent a potential vaccine candidate against different *T. gondii* isolates infection, worth further study.

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