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Low-level sequence variation in *Toxoplasma gondii* calcium-dependent protein kinases among different genotypes

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ABSTRACT. The causative agent of toxoplasmosis, *Toxoplasma gondii*, can infect virtually all nucleated cell types of warm-blooded animals. In this study, we examined the sequence variation in calcium-dependent protein kinase 2 (*CDPK2*) genes among 13 *T. gondii* strains from different hosts and geographical locations. The results showed that the lengths of the complete *CDPK2* DNA and cDNA sequences were 3671-3673 and 2136 bp, respectively, and the sequence variation was 0-0.9% among different *T. gondii* strains. Phylogenetic analysis based on the *CDPK2* gene sequences revealed that *T. gondii* strains

of the same genotypes were clustered in different clades. Further analysis of all the other *T. gondii CDPK* genes in genotype I (GT1), II (ME49), or III (VEG) strains indicated the *T. gondii CDPK* gene family is quite conserved, with sequence variation ranging from 0 to 1.40%. We concluded that *CDPK2* as well as all the other *CDPK* genes in *T. gondii* cannot be used as proper markers for studying the variants of different *T. gondii* genotypes from different hosts and geographical locations, but their sequence conservation may be a useful feature promoting them as anti-*T. gondii* vaccine candidates in further studies.

Key words: *Toxoplasma gondii*; Calcium-dependent protein kinases; Sequence variation; Genotypes

INTRODUCTION

The apicomplexan *Toxoplasma gondii* is an obligate intracellular pathogen that can infect virtually all warm-blooded animals and humans, causing toxoplasmosis (Dubey, 2010; Zhou et al., 2011; Robert-Gangneux and Dardé, 2012). *T. gondii* infection is normally asymptomatic but can cause serious health problems in congenitally infected newborns and immune-compromised individuals (Luft and Remington, 1992; Boyer et al., 2005). Toxoplasmosis in animals, especially in pigs and goats, can cause infertility, spontaneous abortion, and neonatal malformations, which lead to serious economic impacts on livestock industries (Bhopale, 2003). Importantly, consumption of raw or undercooked meat containing *T. gondii* tissue cysts is the main source of human infection and thus raises public health concerns (Tenter et al., 2000).

Calcium-dependent protein kinases (CDPKs) are widely distributed, and found in many kinds of plants, ciliates, and apicomplexan parasites, but are absent in animals and humans (Harper and Harmon, 2005). CDPKs are, however, abundant in apicomplexans (Nagamune and Sibley, 2006). *T. gondii*, for example, was estimated to possess 12 CDPK isoforms (Billker et al., 2009), which have been shown to control diverse important biological functions (Lourido et al., 2011, 2012; McCoy et al., 2012; Morlon-Guyot et al., 2014). In plants, *CDPK* genes are highly conserved (Cheng et al., 2002), but little is known regarding the sequence variation of members of *CDPK* family genes among different *T. gondii* strains. Therefore, the present study was designed to investigate sequence diversity in *CDPK* genes among *T. gondii* strains from different genotypes.

MATERIAL AND METHODS

T. gondii isolates

A total of 13 *T. gondii* strains stemming from different hosts and geographic locations were used for analysis in this study (Table 1). All the *T. gondii* strains have been genotyped in previous studies and their genomic DNA was prepared as described previously (Zhou et al., 2009, 2010; Su et al., 2010; Huang et al., 2012).

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

Strain	Host	Geographical origin	Genotype
RH	Human	France	Reference, Type I, ToxoDB #10
GT1	Goat	United States	Reference, Type I, ToxoDB #10
PTG	Sheep	United States	Reference, Type II, ToxoDB #1
CTG	Cat	United States	Reference, Type III, ToxoDB #2
MAS	Human	France (Reference)	Reference ToxoDB #17
Prugniaud (PRU)	Human	France	Type II, ToxoDB #1
TgC7	Cat	Guangzhou, Guangdong, China	ToxoDB #9
PYS	Pig	Panyu, Guangdong, China	ToxoDB #9
TgCgCa1	Cougar	Canada	Reference, ToxoDB #66
TgCatBr5	Cat	Brazil	Reference, ToxoDB #19
FOU	Human	France	Reference, ToxoDB #6
VEG	Human	United States	Reference, ToxoDB #2
ME49	Sheep	United States	Type II, ToxoDB #1

Polymerase chain reaction (PCR) amplification of *CDPK2* genes

The entire genomic sequence of the *CDPK2* genes from individual *T. gondii* strains was amplified using the oligonucleotide primers FCDPK2 (forward primer: 5'-ATG CCG CTC AAG ACT TCC TGG CAT T-3') and RCDPK2 (reverse primer: 5'-TTA CCC CGT AGC GCG AGG CGT CAG ACT G-3'). The amplification reaction was carried out in a 25-μL total volume, including 12.5 μL Premix Ex Taq (TaKaRa Bio, Otsu, Shiga, Japan), 0.2 μM of each primer, and 100-200 ng template DNA. Amplification of DNA samples from individual strains was carried out in a thermocycler (Bio-Rad, Hercules, CA, USA) under the following conditions: denaturation at 94°C for 10 min (initial denaturation), followed by 35 cycles of 94°C for 50 s (denaturation), 67°C for 40 s (annealing), 72°C for 3 min and 40 s (extension), and a final extension step at 72°C for 10 min. The successful PCR amplifications were confirmed by electrophoresis on a 1% (w/v) agarose gel, and stained with GoldenView™ and the DL 5000 marker (TaKaRa) to estimate the sizes of the *CDPK2* PCR products.

DNA sequencing

PCR products were purified using the PCR-Preps DNA Purification System (Promega, Madison, WI, USA) according to manufacturer recommendations, and then ligated to the pMD18-T vector (TaKaRa). The recombinant vectors were transformed into JM109 competent cells (Promega). Positive recombinant vectors were identified by bacterial PCR amplification directly with the same primers described previously, and sent to Sangon (Shanghai, China) for sequencing.

Sequence analysis and phylogenetic reconstruction

The *CDPK* gene sequences of the VEG, FOU, ME49, and GT1 strains were obtained from the ToxoDB database (http://toxodb.org). All the obtained *T. gondii CDPK* sequences were aligned using the computer program Clustal X 1.81 (Thompson et al., 1997) and then the sequence variation was determined among the examined *T. gondii* strains. Phylogenetic reconstructions based on the complete *CDPK2* gene sequences of different *T. gondii* strains were performed using maximum parsimony (MP), and neighbor joining (NJ) with the outgroup using the corresponding sequence from *Neospora caninum* (GenBank accession No. FR823391.1). MP and NJ analyses were performed using PAUP* 4.0 with indels treated as missing

character states (Swofford, 2002). A total of 1000 random addition searches using tree bisection-reconnection branch swapping were performed for each MP and NJ analysis. Bootstrap probability was calculated from 1000 bootstrap replicates with 10 random additions per replicate in PAUP. The nucleotide composition, transition, tranversion, and the genetic distance calculations were performed using the MegAlign program in the DNA Star Version 5.0 software (Xu et al., 2014).

RESULT AND DISCUSSION

The obtained amplicons of the *CDPK2* genes were determined to be approximately 3600 bp in length by agarose gel electrophoresis for all 10 *T. gondii* strains. The sequence results showed that the complete genomic sequence of the *CDPK2* gene was 3671 bp in length for the strains TgC7 and PYS, 3673 bp in length for MAS and TgCgCa1, and 3762 bp for the others. Comparison of the *CDPK2* genomic sequences using the Gene Structure Display Server (GSDS) (Guo et al., 2007) identified that four introns and five exons were distributed across the *CDPK2* gene. A total of 52 variable nucleotide positions containing 46 nucleotide substitutions, one single nucleotide polymorphism, and five nucleotide losses were identified in the *CDPK2* gene sequences, with intra-specific variations of 1.42% (52/3673) for genomic DNA sequences and 0.66% (14/2136) for cDNA sequences. Fourteen nucleotide substitutions were identified in the expressed regions that were predicted to result in three amino acid substitutions (Table 2). The transitions and transversions in the entire genomic sequences and in the coding region of *T. gondii CDPK2* genes are shown in Table 3, which indicated that the intra-specific nucleotide variations occurred primarily in introns.

The phylogenetic reconstruction based on *CDPK2* sequences from 13 *T. gondii* strains showed that the same genotypes were not clustered in a clade (Figure 1), which was similar to previous results from our group of phylogenetic reconstruction using *PLP1* (Yan et al., 2011), *ROP7* (Zhou et al., 2012), *eIF4A* (Chen et al., 2014), *MIC13* (Ren et al., 2011), and *ROP38* (Xu et al., 2014). The results from this analysis suggested that the *T. gondii CDPK2* gene would not be an ideal genetic marker for intra-species phylogenetic analysis to identify different genotypes of *T. gondii* isolates from different hosts and geographical locations because of its low variation.

Further analysis of the other *T. gondii CDPK* genes based on the genomic sequences available in the ToxoDB database showed similar low variation of *CDPK* genes among *T. gondii* genotypes I, II, and III in general, with the highest genetic distance found in *TgCDPK6* (1.4%) and the lowest in *TgCDPK1 4* and *4A* genes (0%) (Table 3). The low variation of *CDPK* genes suggested conserved functionality for each *CDPK* gene in different *T. gondii* strains as well. In previous studies, *TgCDPK1*, *TgCDPK3*, and *TgCDPK7* were shown to participate in parasite motility, such as host-cell invasion and egress, and parasite division and growth (Lourido et al., 2011, 2012; McCoy et al., 2012; Morlon-Guyot et al., 2014). The important functions and the low variation of CDPKs in different *T. gondii* strains imply that they may be considered promising vaccine candidates, as the protective immunity induced by *T. gondii* CDPK3 revealed in our previous study showed the suitability of this protein family for development of new vaccines against *T. gondii* parasite infection (Zhang et al., 2013).

In conclusion, the present study revealed the low variability in *CDPK2* gene sequences among *T. gondii* isolates from different geographical regions and hosts and further investigated the sequence variation in all *CDPK*s among different *T. gondii* genotypes. This analysis showed that the *CDPK* gene family in *T. gondii* is very conserved, which suggests that *T. gondii CDPK* genes may be useful as candidate vaccines against toxoplasmosis.

Table 2. Characteristics of *Toxoplasma gondii CDPK2* gene sequences including their introns and exons.

Items	*CDPK2* DNA	CDPK2 CDS	*CDPK2* intron				*CDPK2* exon				
			First	Second	Third	Fourth	First	Second	Third	Fourth	Fifth
Length	3671-3673	2136	448-449	445-446	268-270	371-372	739-739	482-482	298-298	376-376	241-241
G+C%	54.34-54.49	57.49-57.77	52.01-52.9	49.56-47.53	48.88-49.26	51.61-52.15	58.19-58.59	57.05-57.88	57.38-56.38	54.79-55.05	59.75-59.75
Transition	29	9	7	7	2	4	2	4	2	1	0
Transversion	17	5	7	3	1	1	1	2	0	0	2
R	1.71	1.80	1.00	2.33	2.00	4.00	2.00	2.00	-	-	-
Distance %	0-0.9	0-0.5	0-2.0	0-1.8	0-1.5	0-1.4	0-04	0-0.8	0-0.7	0-0.3	0-0.8

R = transition/transversion; CDS = coding sequence; Distance = divergence between two genes (if the value is higher the divergence is larger).

Table 3. Characteristics of *Toxoplasma gondii CDPK* gene sequences.

Items	CDPK2B[a]	CDPK3[a]	CDPK5[a]	CDPK9[a]	CDPK1[b]	CDPK2A[b]	CDPK4[b]	CDPK4A[b]	CDPK6[b]	CDPK7[b]	CDPK8[b]
Length DNA	5547	7556-7593	5260-5267	14608-14649	5356-5359	6718-6737	12405-12413	14749-14771	11131-11398	10872-10874	8435-8463
Length CDS	1815	1614	2046	1995-2001	1749	2361	3477	1632	4434	6402	4497
G+C%	48.72-48.78	48.04-48.25	53.33-53.88	48.73-48.82	54.52-54.54	52.12-52.25	51.84-51.87	50.20-50.27	49.93-50.53	57.76-57.83	55.71-55.87
Transition	6	38	47	110	0	48	1	0	103	38	46
Transversion	7	24	31	59	0	34	0	0	49	27	37
Loss	2	43	2	61	0	23	24	26	305	6	18
VN	15	105	80	230	2	105	25	26	457	71	101
AA mutation	2	0	0	2	0	2	0	0	15	10	12
R	0.86	1.58	1.51	1.86	-	1.41	-	-	2.10	1.40	1.24
Distance	0-0.2	0-0.8	0.6-0.8	0.1-1.1	0	1.2	0	0	1.4	0.6	1

[a]Gene sequences from genotype I (GT1), II (ME49), and III (VEG) strains; [b]gene sequences from genotype I (GT1) and II (ME49) strains. VN = variable nucleotide; R = transition/transversion; CDS = coding sequence; AA = amino acid. Distance = divergence between two genes (if the value is higher the divergence is larger).

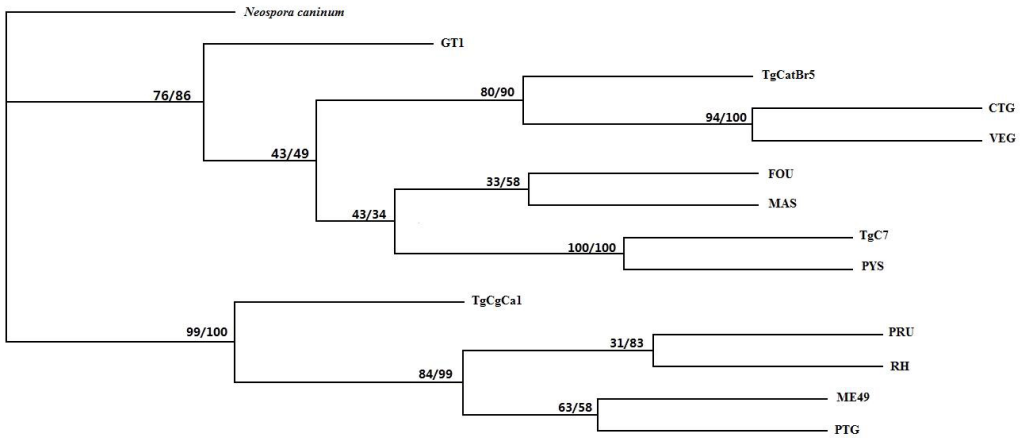

Figure 1. Phylogenetic relationships of *Toxoplasma gondii* isolates from different hosts and geographical locations. Relationships were constructed by maximum parsimony (MP) and neighbor-joining (NJ) analyses based on the *TgCDPK2* gene sequences using *Neospora caninum* (GenBank accession No. FR823391.1) as the outgroup. Numbers of MP/NJ bootstrap values (%) are indicated along the branches.

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