Seroprevalence and Phylogenetic Analysis of *Toxoplasma gondii* from Domestic Cats, Captive Wild Felids, Free-range Wild Felids and Rats in Certain Regions of Thailand

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Abstract

Toxoplasmosis is an important zoonotic disease caused by *Toxoplasma gondii*, an obligated zoonotic apicomplexan parasite. The infection varies according to geographical areas. This work aimed to study the seroprevalence and genotype of *T. gondii* infection in domestic, captive and free-range wild felids, and in their small mammal prey, rats (*Rattus spp*). Two hundred and ninety three sera, received from the 4 individual animal groups in Thailand, were tested using the indirect latex agglutination test (ILAT) for specific antibody detection. The nested-PCR for *glycerol-3-phosphate* (*B1*) and *bradyzoite surface antigen* (*SAG4*) gene detection was used to detect seropositive animals and PCR product was submitted for DNA sequencing. Out of the 293 sera, ILAT showed 11.68% positive results. *T. gondii* were found 3.48% seropositive in the domestic cats (n=86), 18.84% seropositive in the captive wild felids (n=138), 14.28% seropositive in the free-range wild felids (n=7), and 6.67% seropositive in the murine prey (n=60). Tissues from the seropositive animals such as liver, heart, brain and skeletal muscle were collected, and then DNA was extracted to perform nested-PCR and sequence analysis. By the nested-PCR, the brain and muscle tissues received from 3 black rats and a clouded leopard (1.37%) were found positive for *T. gondii*. *SAG4* and *B1* might serve as novel genetic markers for population genetic studies of *T. gondii* isolates. Based on the ML phylogenetic tree analysis of *SAG4* and *B1* coding sequences, *T. gondii* found in 3 murine prey and a clouded leopard was close to *T. gondii* RH type I strain with approximately 99-100% similarity. This is the first report on the relation of *T. gondii* infection with strain identification in domestic cats, captive and free-range felids, and murine in Thailand. Better understanding of the genetic diversity will lead to better management, prevention and treatment of this disease in the valuable species of wild felids.

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Introduction

Toxoplasma gondii is an obligated zoonotic apicomplexan parasite most extensively studied for the tissue encysting coccidian. *T. gondii* infects virtually all warm-blooded animals worldwide. It has a two-host life cycle with felids as the definitive host in which sexual replication of the parasite occurs. It has a wide range of intermediate hosts, almost all are warm-blooded animals, including mammal, rodent, avian, and human. *T. gondii* belongs to the phylum *Apicomplexa*, which includes other important parasitic protozoan in the genus *Plasmodium*, *Coccidia*, *Cyclospora*, *Cryptosporidium*, *Neospora* and *Babesia* (Mc Conkey et al., 2013). In human population, the infection varies according to geographical areas. The seroprevalence in humans ranged from 2.3% to 90% worldwide (Sukthana, 2006). It could be stated that one third of the human population has been infected (Kim and Weiss, 2008). The difference in geographical area creates different types of *T. gondii*, as a result, the disease and clinical signs are different in different areas (Kikuchi et al., 2004; Zakimi et al., 2006). The population structure of *T. gondii* in different parts of the world, in Europe, Africa and North America, shows low genetic diversity associated with 3 main lineages. Even though this parasitic protozoan has only one species in the genus *Toxoplasma*, different genotypes can be differentiated from clinical signs in laboratory mice and difference in genetic marker (Dardé, 2004). For molecular detection, the *glycerol-3-phosphate dehydrogenase* (*B1*) gene is the species-specific sequence frequently used for the detection of this parasite by Burg and colleagues (1989), even though the function of this gene has not yet been known and several other single-copy sequences including the *bradyzoite surface antigen* (*SAG1*, *SAG2*, *SAG3*, *SAG4*) genes and the *organite antigen* (*GRA4*) gene have also been used as DNA amplifying targets (Switaj et al., 2005).

Strains of *T. gondii* have been classified into three genotypes, types I, II, and III, based on the polymorphisms of their genes (Sibley and Boothroyd, 1992b). Type I is considered as the dominant type in Europe and North America, whereas type III is reported in Asia. In Africa, most of the dominant types reported were non-archetypal lineage (Howe and Sibley, 1995). Type I strain differs genetically by approximately 1 percent from type II and type III strains (Sibley and Boothroyd, 1992a, 1992b). Type I strain led to widespread parasitic dissemination and death of mice less than 10 days after inoculation of < 10 tachyzoites. In contrast, mice could survive from infection with a type II strain (LD_{50} > 10^3) and their tachyzoite dissemination was much less extensive. Type III infection is the cause of progressive deterioration and death of mice. It was notable with neurological symptoms which can occur a few weeks or months after infection (Robben et al., 2004; Saeij et al., 2005). A study of migration across the extracellular matrix of mammalian cells showed that type I and type III had high migratory capacity, revealing long-distance migration and linking to acute virulence (Barragan and Sibley, 2003). However, the determinants which dramatically affect the virulence of different *T. gondii* strains in hosts and the pathogenesis of toxoplasmosis are only partially understood. The genotype of *Toxoplasma* parasites may influence the development of clinical illness in hosts (Miller et al., 2000; Mordue et al., 2001).

In one health triad, wildlife was claimed as a source of infectious diseases that threaten human. A spillover of parasite or diseases from wildlife to human or vice versa was proved as wildlife has harbored some diseases. The change in the ecosystem which they live in, and also human activities, influence the flow of parasite or diseases. The seroprevalence of *T. gondii* and sequence analysis results in this study will give us information about *T. gondii* characteristic, parasite behavior, and pathogenesis, leading to effective prevention, treatment and disease control. This study focused on the seroprevalence study in domestic cats, captive and free-range wild felids and murine prey nearby these felid's habitat areas which acted as prey for predators. Moreover, genetic characterization of *T. gondii* isolated from hosts would be characterized based on the *B1* and *SAG4* genes. In Thailand, information about genetic variation in these animals is scarce, therefore, this study was conducted according to the seroprevalence of *T. gondii* and with genetic characterization. Molecular technique using nested-PCR was used to detect *T. gondii* DNA in animal tissue carcasses, and then DNA sequences were analyzed for phylogenetic study.

Materials and Methods

Animal samples and specimen collection: Sample collection of captive and free-range wild felids and their murine prey was conducted during 2007-2010 in several regions of Thailand; most of the samples were from the central and western regions. Blood samples were collected from 140 captive wild-felids. These captive wild felids were composed of 30 leopard cats (*Prionailurus viverinnus*), 4 Asian golden cats (*Captopuma temminckii*), 12 leopards (*Panthera pardus*), 75 tigers (*Panthera tigris*), 5 clouded leopards (*Pardofelis nebulosa*), 4 jungle cats (*Felis chaus*), 2 flat-headed cats (*Prionailurus planiceps*), 4 lions (*Panthera leo*) and 4 cougars (*Puma concolor*). They belonged to 2 wildlife breeding centers in Ratchaburi province, 2 government zoos located in Chonburi and Suphanburi provinces, and 2 private zoos located in Kanchanaburi and Nakhon Nayok provinces in central Thailand (Figure 1 and Table 2). Blood samples of 7 free-range fishing cats (*Prionailurus viverinnus*) were provided by Department of National Parks, Wildlife and Plants from previous studies of the fishing cat's habitat and heath checking program in Khao Sam Roi Yod Natural Park, Prachuap Khiri Khan province, Thailand. Sixty rats (*Rattus sp*), representative samples of prey, were captured by using traps from places nearby in Kanchanaburi, Ratchaburi, Phichit and Phitsanulok provinces. Blood specimens were collected by tail-vein puncture and then the rats were sacrificed. Moreover, blood samples were collected from 86 healthy domestic cats (*Felis domesticus*) by random sampling from cats coming to the Prasu-Arthorn Veterinary Teaching Hospital, Mahidol University, Nakhon Pathom province (from areas of Bangkok metropolis and its vicinity), and 6

private veterinary clinics located in Nakhon Pathom, Kanchanaburi, and Chiang Mai provinces under owners' consent forms (Figure 1 and Table 2).

Prior to blood collection, the large and middle wild felids were anesthetized with 10 mg/kg dosage of Tiletamine/Zolazepam (Zoletil®, Virbac laboratories, France). The small wild felids were also sedated with 0.5 mg/kg dosage of xylazine hydrochloride (Rumpun®, Bayer, Ansan, Korea) sedative drug, before being caught by a hand-net. Five milliliters of whole blood from each animal was collected and then aliquoted in an EDTA blood collection tube (BD Vacutainer® SST™; BD Diagnostic Systems, U.S.A.) and a sterile tube. For specific *Toxoplasma*-antibody

testing, blood was collected into BD Vacutainer® SST™ (BD Diagnostic Systems, U.S.A.) and serum was separated by centrifugation at 1,200 g. The sera were transferred to a new tube and stored at -80°C until analysis. Organ specimens were collected from dead captive wild felids and murine prey, and stored in deep freezer (-80°C) until analysis. The practices with animal in this study (Protocol No. MUVS 2009-05) were approved by the Institutional Animal Care and Use Committee based on "Guide for the care and use of agricultural animals in agricultural research and teaching" (FASS, 2010) and "Guidelines of the American society of mammalogists for the use of wild mammals in research" (Sikes et al., 2011).
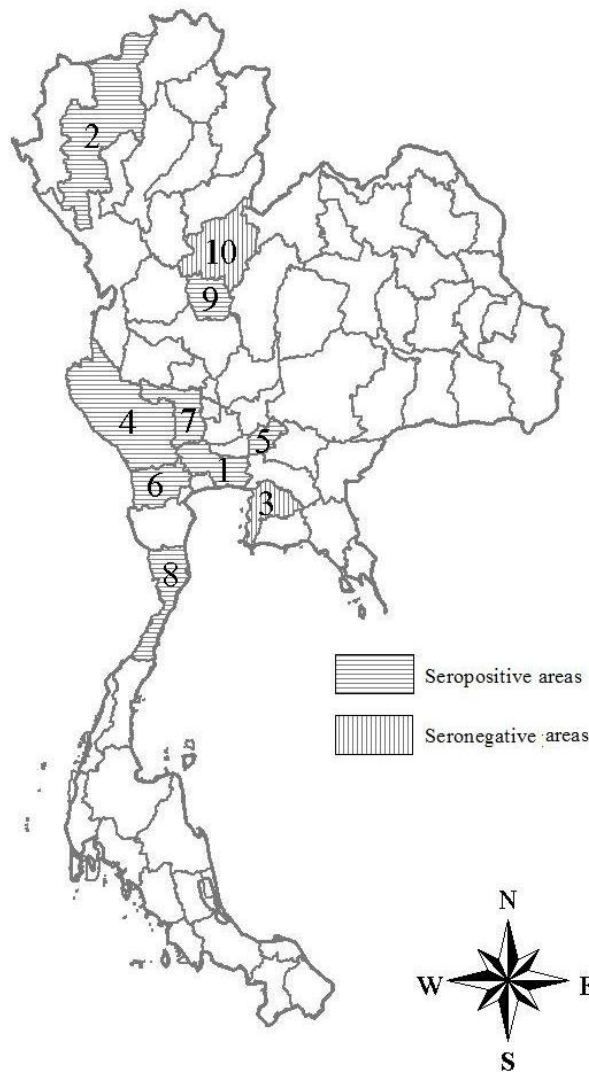
Figure 1 Map showing locations of *T. gondii* studied in felids and murine in Thailand during 2007-2010. The numbers represent the studied areas in 10 provinces: 1. Bangkok metropolis and its vicinity, 2. Chiang Mai, 3. Chonburi, 4. Kanchanaburi, 5. Nakhon Nayok, 6. Ratchaburi, 7. Suphanburi, 8. Prachuap Khiri Khan, 9. Phichit, and 10. Phitsanulok.

Table 1 Oligonucleotide primers used in nested-PCR

Specific gene	Primers	Sequences (5′ to 3′)	Length of amplicon (bps)	Application
B1[*]	B1-ext-F	TGTTCTGTCCTATCGCCAACG		amplifying
	B1-ext-R	ACGGATGCAGTTCCTTTCTG		
	B1 int F	TCTTCCCAGACGTGGATTTC	516	amplifying/
	B1 int R	CTCGACAATACGCTGCTTGA		sequencing
SAG4[**]	SAG4-ext-F	TACGATTTCAAGAAGGCGCT		amplifying
	SAG4-ext R	ACGGATGCAGTTCCTTTCTG		
	SAG4-int-R	GGACGACGATGAAACAAAGA	169	amplifying/
	SAG4 –int-R	CAGAAGGGTATGGTTTCCTTCT		sequencing

[*]Primers specific to *B1* gene were described by Grigg and Boothroyd (2001); [**]Primers specific to *SAG4* gene were designed in this present study.

Table 2   ILAT serological test and nested-PCR detection of *T. gondii* in felids and murine prey in 10 provincial areas in Thailand

Species of animal subjects in provincial levels	Group of subjective animals	No. of animals	ILAT positive	Nested-PCR positive
Bangkok metropolis and its vicinity				
domestic cats (*Felis domesticus*)	D	69	3	0
Chiang Mai				
domestic cats (*Felis domesticus*)	D	17	1	0
Chonburi				
jungle cats (*Felis chaus*)	CW	4	0	0
cougar (*Puma concolor*)*	CW	4	0	0
Kanchanaburi				
leopards (*Panthera pardus*)	CW	1	0	0
lions (*Panthera leo*)**	CW	2	0	0
tigers (*Panthera tigris*)	CW	47	4	0
rats (*Rattus sp*)	P	7	0	0
Nakhon Nayok				
tigers (*Panthera tigris*)	CW	14	1	0
Ratchaburi				
Asian golden cats (*Captopuma temminckii*)	CW	4	3	0
clouded leopard (*Pardofelis nebulosa*)	CW	5	2	1
flat-headed cats (*Prionailurus planiceps*)	CW	2	0	0
leopards (*Panthera pardus*)	CW	9	4	0
leopard cats (*Prionailurus bengalensis*)	CW	30	3	0
rats (*Rattus sp*)	P	32	2	2
tigers (*Panthera tigris*)	CW	11	8	0
Suphanburi				
leopards (*Panthera pardus*)	CW	2	0	0
lions (*Panthera leo*)**	CW	2	0	0
tigers (*Panthera tigris*)	CW	3	1	0
Prachuap Khiri Khan				
fishing cats (*Prionailurus viverinnus*)	FW	7	1	0
Phichit				
rats (*Rattus sp*)	P	12	1	1
Phitsanulok				
rats (*Rattus sp*)	P	9	0	0
Total		**293**	**34**	**4**

D = domestic animal; FW = free-range wild animal; CW = captive wild animal; P = prey animal; *natural habitat in America; **natural habitat in Africa and a part of Asia

Table 3   ILAT serological test and nested-PCR detection of *T. gondii* in felids and murine prey in 10 provincial areas in Thailand

Groups	Common names (Species)	No. of animals	ILAT positive in each titer	
			1:128	1:256
domestic felids	domestic cats (*Felis domesticus*)	86	4	0
free-range felids	fishing cats (*Prionailurus viverinnus*)	7	0	1
captive wild felids	leopard cats (*Prionailurus bengalensis*)	30	3	0
	Asian golden cats (*Captopuma temminckii*)	4	3	0
	leopards (*Panthera pardus*)	12	3	1
	tigers (*Panthera tigris*)	75	12	2
	clouded leopard (*Pardofelis nebulosa*)	5	2	-
	flat-headed cats (*Prionailurus planiceps*)	2	0	0
	jungle cats (*Felis chaus*)	4	0	0
	cougar (*Puma concolor*)*	4	0	0
	lions (*Panthera leo*)**	4	0	0
	total captive felids	**140**	**27**	**4**
murine prey	rats (*Rattus sp*)	60	3	0
Total		**293**	**34**	

*natural habitat in America; ** natural habitat in Africa and a part of Asia

Serology tests: Specific anti-*T. gondii* antibodies were tested by the indirect latex agglutination test (ILAT, ToxoCheck™, Eiken, Japan). ILAT was performed in U-bottomed 96-well microtiter plates according to the manufacturer's recommendation. Briefly, the agglutination was done in microtiter plates (Nunc™, N.Y., U.S.A.). A volume of 25 µl of buffer (0.2M 2 amino-2 methyl-1 propanol-HCl buffer solution) was added to each well. Then, 25 µl of the test sera, which were serially diluted from 1:16 to 1:2,048, were charged to each well followed by 25 µl of the sensitive latex suspension. The plates were gently shaken, properly mixed and then sealed. Then, reactions were allowed to stand for 12 hrs at room temperature. The positive and negative control sera were also maintained in each plate. Agglutination that was intense with irregular edges and spread uniformly throughout the well was interpreted as a positive reaction ≥ 64 titer while a distinct circular precipitation in the center indicated a negative reaction.

DNA extraction and nested-PCR: According to the indication of the manufacturer (QIAmp® blood and tissue kit, Qiagen™, Germany), DNA was extracted from the blood samples collected from domestic cats, captive wild felids and rats. The DNA was stored in -20°C until used. Four pairs of oligonucleotide primers and amplifying parameter of the *glycerol-3-phosphate dehydrogenase* (*B1*) gene followed a previous study by Griggs and Boothroyd (2001) (Table 1). The outer and inner primer pairs for *SAG4* gene amplification were designed in this present study. Five *bradyzoite surface antigen 4* (*SAG4)* coding sequences available from GenBank (AF340224-AF340226, Z69373 and XM_002371410) were used for multiple alignments using BioEdit ver.7.2.5 package. Consensus sequences were selected for primer design (Table 1). Nested-PCR reactions were performed in 50 µl of a solution containing 5 µl of extracted DNA and 45 µl of 0.4 pmol of each primer, 300 µM of each dNTP (Qiagen™, Germany), 4 units of HotStarTaq™ DNA Polymerase (Qiagen™, Germany), 1x PCR buffer (20 mM Tris–HCl pH 8.4, 50 mM KCl), 1.5 mM MgCl₂ and nuclease-free sterile water. Amplification was performed in a thermocycler (PTC-200, MJ Research™, U.S.A.) and thermocycling consisted of one step of 5 min at 95°C followed by 35 cycles of 45 sec at 94°C, 60 sec at 56°C, and 90 sec at 72°C with a final extension step of 7 min at 72°C. Sizes of the amplified DNA fragments were determined in 1.5% agarose gel electrophoresis compared with a 100-bp ladder (New England Biolabs™, Hertfordshire, U.K.) followed by staining with GelRed® fluorescence (Biotium™, U.S.A.) and visually recorded under a UV-transilluminator.

Phylogenetic tree analysis: The DNA amplicons from each nested-PCR reaction were purified by QIAquick gel extraction kit (Qiagen, Germany). Direct sequencing of the amplicons was determined by the di-deoxyribonucleotide dye terminator method using BigDye® terminator v3.1 cycle sequencing kit (Applied Biosystem, Carlsbad, CA., U.S.A.) according to instructions from the supplier. The sequencing reactions were performed by 25 cycles of 94°C for 30 sec, 50°C for 15 sec, and 60°C for 4 min. The internal

primers of each *SAG4* and *B1 ge*nes were used as sequencing primer for nucleotide analysis. Thereafter, the purified DNA amplicons were precipitated with absolute Ethanol and 3M Sodium acetate and subjected to ABI.3730XL automatic sequencers (Applied Biosystems, Waltham, MA., U.S.A.) to read the sequences. Each gene sequence fragment was assembled by BioEdit (version 7.2.5) program. The maximum-likelihood (ML) method from the MEGA (version 5.2) program was applied to construct phylogenetic trees of the aligned sequences. Bootstrap analyses were performed on the ML trees with 1,000 pseudo-replicates.

Results

ILAT serology test: The result of *T. gondii*-specific antibody in animals is shown in Table 2. The seroprevalence of *T. gondii* infection in 11 provinces ranged from 0% to 29.03%. The *T. gondii*-seropositive animals were found in 8 provincial areas including Bangkok metropolis and its vicinity (n=69), Chiang Mai (n=17), Kanchanaburi (n=57), Nakhon Nayok (n=14), Ratchaburi (n=93), Suphanburi (n=7), Prachuap Khiri Khan (n=7), and Phichit (n=12). The percentages of seropositive animals/number of tested samples in these 8 provinces were 4.35%, 5.88%, 7.02%, 7.14%, 29.03%, 14.29%, 14.29%, and 8.33%, respectively. There were 2 provincial areas, Chonburi (n=8) and Phitsanulok (n=9), where no animals were found seropositive (Figure 1 and Table 2). Thirty-four of the total of 293 animals were seropositive for *T. gondii* infection, equivalent to 11.60% of prevalence. The seroprevalence by ILAT testing in the domestic felids (n=86), free-range felids (n=7), captive wild felids (n=140) and murine prey (n=60) were found at 4.65%, 14.29%, 22.14%, and 5.0% respectively. Most of the seropositive titer in each animal group occurred at a low level ranging from 1:64 to 1:256 (Table 3).

Nested-PCR detection: The positive DNA band of nested PCR reactions showed 516 base pairs in length (Figure 2). The study found that none of the 86 domestic cats and 7 free-range felids were positive by the nested-PCR detection. One of the 138 captive felids (14.29%) was found positive by the nested-PCR. *T. gondii* DNA was detected in the brain-tissue samples from a carcass of a captive clouded leopard in Ratchaburi province (isolate MU3). Moreover, 2 positive specimens (isolates MU1 and MU2) were found in the brain tissues of 2 trapped black rats from the places nearby Ratchaburi province. Another positive specimen (isolate MU4) was collected from the muscle tissues of trapped black rats in Phichit province (Table 2). The positive cases of *T. gondii* based on the nested-PCR detection were 1.37% (4 of 293) from the total of 293 animals and 11.76% (4 of 34) in the seropositive animals.

Phylogenetic tree analysis: The 4 *T. gondii SAG4* coding sequences (229 bps) in this study were named as MU1, MU2, MU3, and MU4 strains (KF425009, KF425010, KF425011 and KF425012, respectively). Sequences were aligned and compared with *SAG4* coding sequences of RH type I strain (AF340224),

Prugniaud type II strain (AF340225), ME49 type II strain (XM_002371410), CEP type III strain (AF340226) and PLK strain (Z69373). The bradyzoite protein (*SAG4*) coding sequence of *Neospora caninum* (AY763105) was used for outgroup comparison. The similarities of *SAG4* coding sequences were 100% identical to RH type I strain, ME49 type II strain, and

Prugniaud type II strain. The phylogenetic analysis using ML based on the *SAG4* coding sequences showed that the 4 Thai *T. gondii* strains were grouped into a separate cluster of type I and type II and that the *T. gondii* CEP type III strain could be readily distinguished from the other genotypes (Figure 3).
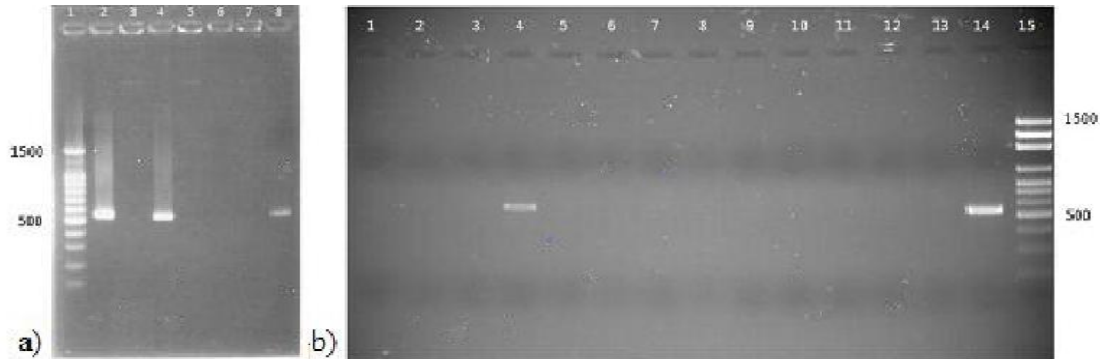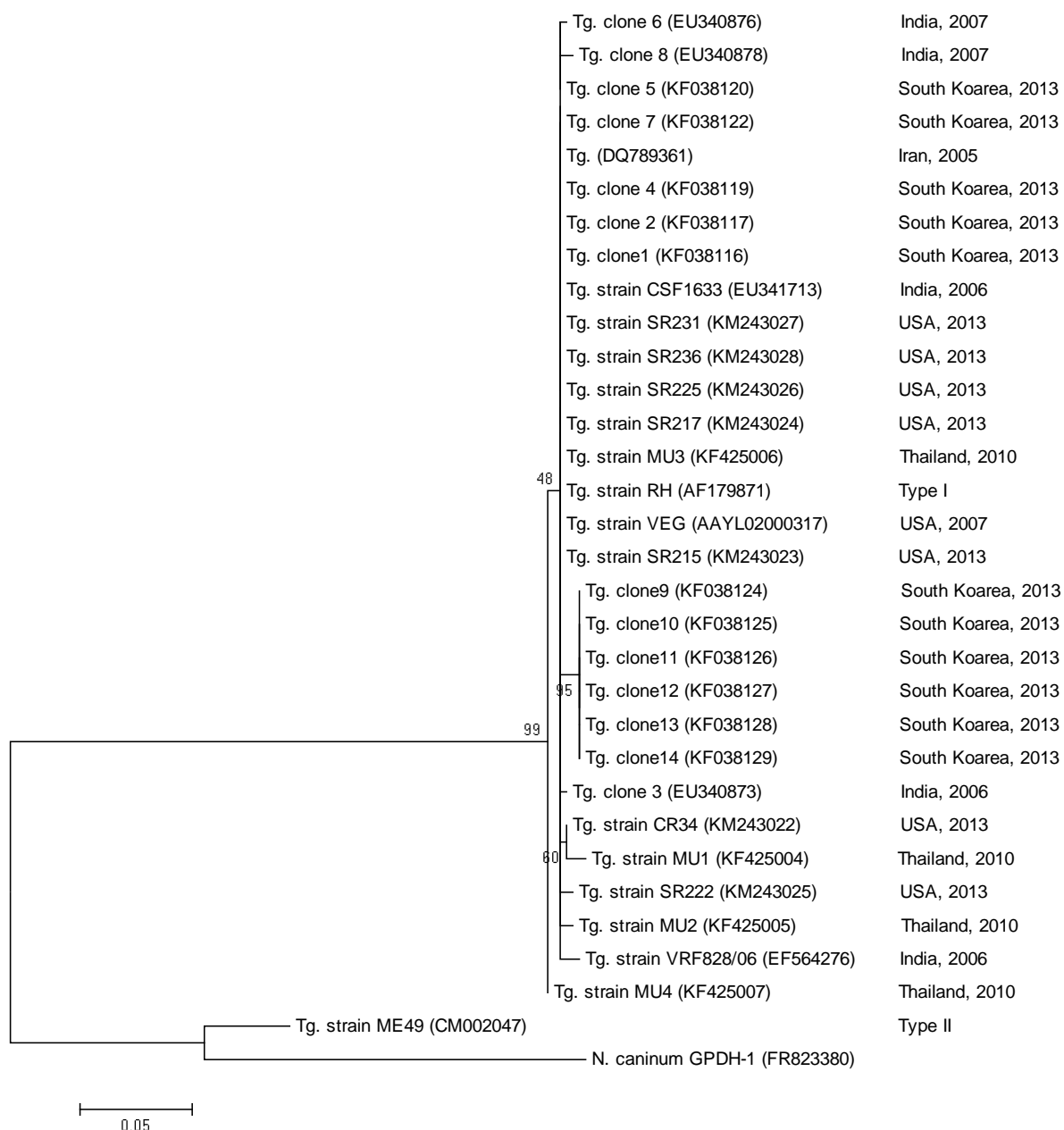

Figure 2   Electophoretogram demonstrating nested-PCR amplicons of *T. gondii B1* gene. **a)** DNA amplicon fragment (516 bps) found in black rat's brain sample (lane 4); Lane 1, 100 base pair standard DNA ladder; Lane 2, positive control; Lanes 5-7, muscle specimens collected from black rats showing negative amplifying; Lane 8, muscle specimens collected from black rats showing positive fragment (516 bps). **b)** DNA amplicon fragment found in clouded leopard's brain specimen (lane 4); Lanes 1-3, specimens from domestic cats, Lanes 6-10, specimens from captive tigers; Lane 11, first round negative control; Lane 12, second round negative control; Lane 13, positive control; Lane 14, 100 base pair DNA ladder.
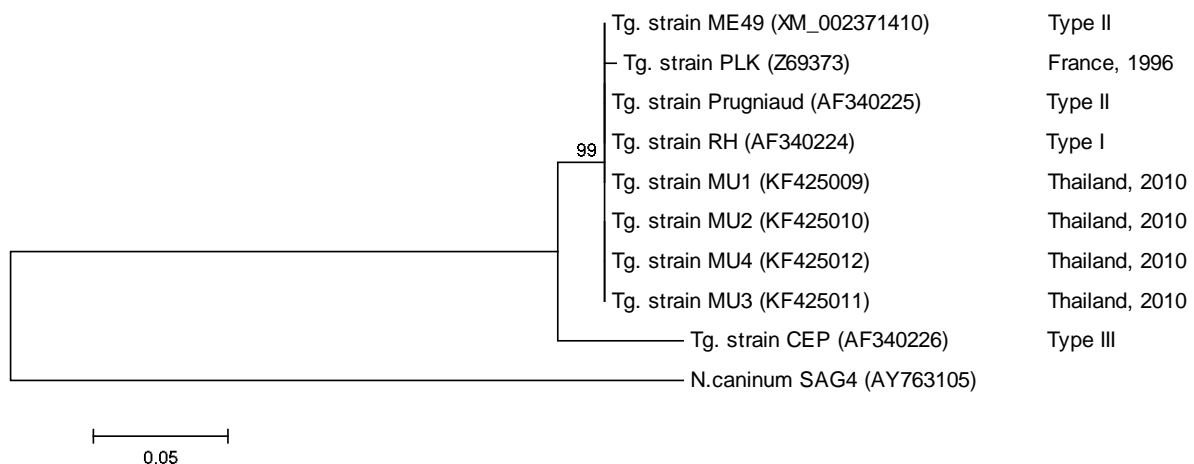
Figure 3   Phylogram of 4 Thai *T. gondii* isolates determined by analysis of *SAG4* gene and compared with prototype strains belonging to genotypes I, II and III. Phylogenetic relationships were constructed by maximum-likelihood (ML) using *Neospora caninum* as the outgroup.

On the other hand, the *T. gondii B1* coding sequences from 3 isolates from the black rats in the present study were submitted to the GenBank database as MU1, MU2 and MU4 strains (KF425004, KF425005 and KF425007, respectively). Another sequence from the clouded leopard, as MU3 strain (KF425006), was also submitted. These 4 putative *B1* coding sequences (358 bps) were compared with other *B1* coding sequences from RH type I strain (AF179871), ME49 type II strain (CM002047) and other 25 non-typing *T. gondii* available in the GenBank database (AAYL02000317, DQ789361, EF564276, EU341713, EU340873, EU340876, EU340878, KF038120, KF038122, KF243025, KF38116, KF38117, KF38119, KF038124 - KF038129, KM243022 - KM242024 and KM243026 - KM243028). The *B1* sequence of *T. gondii* type III was

not available for access from the GenBank. A sequence of *glycerol-3-phosphate dehydrogenase 1* (*GPDH-1*) gene of *Neospora caninum* (FR823380) similar to *T. gondii B1* gene was used for outgroup comparison. The nucleotide alignment showed that RH type I strain was highly identical (99% identity) to the Thai *T. gondii B1* coding sequence. The phylogenetic analysis using ML based on the *B1* coding sequences showed that all 4 Thai *T. gondii* strains were grouped into a separate cluster of type I, similar to the *T. gondii* isolated from humans in India (Mahalakshmi et al., 2007), aborted sheep in Iran (Habibi et al., unpublished data), rabbits in South Korea (Shin et al., 2013), and California mussels in U.S.A. (Shapiro et al., 2014). The *T. gondii* ME49 type II strain could be readily distinguished from the other genotypes (Figure 4).

Tg. clone 6 (EU340876)	India, 2007	
Tg. clone 8 (EU340878)	India, 2007	
Tg. clone 5 (KF038120)	South Koarea, 2013	
Tg. clone 7 (KF038122)	South Koarea, 2013	
Tg. (DQ789361)	Iran, 2005	
Tg. clone 4 (KF038119)	South Koarea, 2013	
Tg. clone 2 (KF038117)	South Koarea, 2013	
Tg. clone1 (KF038116)	South Koarea, 2013	
Tg. strain CSF1633 (EU341713)	India, 2006	
Tg. strain SR231 (KM243027)	USA, 2013	
Tg. strain SR236 (KM243028)	USA, 2013	
Tg. strain SR225 (KM243026)	USA, 2013	
Tg. strain SR217 (KM243024)	USA, 2013	
Tg. strain MU3 (KF425006)	Thailand, 2010	
Tg. strain RH (AF179871)	Type I	
Tg. strain VEG (AAYL02000317)	USA, 2007	
Tg. strain SR215 (KM243023)	USA, 2013	
Tg. clone9 (KF038124)	South Koarea, 2013	
Tg. clone10 (KF038125)	South Koarea, 2013	
Tg. clone11 (KF038126)	South Koarea, 2013	
Tg. clone12 (KF038127)	South Koarea, 2013	
Tg. clone13 (KF038128)	South Koarea, 2013	
Tg. clone14 (KF038129)	South Koarea, 2013	
Tg. clone 3 (EU340873)	India, 2006	
Tg. strain CR34 (KM243022)	USA, 2013	
Tg. strain MU1 (KF425004)	Thailand, 2010	
Tg. strain SR222 (KM243025)	USA, 2013	
Tg. strain MU2 (KF425005)	Thailand, 2010	
Tg. strain VRF828/06 (EF564276)	India, 2006	
Tg. strain MU4 (KF425007)	Thailand, 2010	
Tg. strain ME49 (CM002047)	Type II	
N. caninum GPDH-1 (FR823380)		

0.05

Figure 4   Phylogram of 4 Thai *T. gondii* isolates determined by analysis of *B1* gene. The *B1* coding sequences were compared with type I and type II prototype strains and 25 *B1* coding sequences of non-typing strains. Phylogenetic relationships were constructed by maximum-likelihood (ML) using *Neospora caninum* as the outgroup.

Discussion

The seroprevalence data of *T. gondii* infection in Thailand are limited. A number of studies of seroprevalence in domestic and wild animals were conducted and results varied from 3.4-71.43%, which are close to the prevalence in this study (Jittapalapong et al., 2005; Buddhirongawatr et al., 2006; Sukthana, 2006; Thiangtum et al., 2006; Jittapalapong et al., 2007; Inpankaew et al., 2010; Jittapalapong et al., 2010; Jittapalapong et al., 2011; Sukhumavasi et al., 2012; Wiengcharoen et al., 2012). The conventional technique for the diagnosis of *T. gondii* infection mainly relies on serological methods such as ELISA, latex agglutination test (LAT), Immunofluorescence assay (IFA) as well as the isolation of *T. gondii* by animal biological assays. In this study, the serologic prevalence was higher in the

captive wild felids (18.84%, n=138) than the free-range wild cats (14.28%, n=7). A previous study in Thailand by Thiangtum and colleagues (2006) reported that the seroprevalence of *T. gondii* infection was 15.4% in captive wild felids maintained in zoo and wildlife breeding centers. Moreover, another study in Thailand (Buddhirongawatr et al., 2006) reported higher seroprevalence (42.8%) by detected seropositive titer in immunocompromised captive wild felids in zoos and wildlife breeding centers. When compared with the seroprevalence in captive wildlife elsewhere, the titer was high in this group of animals as Silva and colleagues (2001) reported that seroprevalence of 64.9% was noted in 71 zoological parks and 15 breeding centers, including nearly all zoos and breeding centers in Brazil. Moreover, even 100% seropositivity was reported by Pas and Dubey (2008).

Reason for the higher titer was the food given to captive animals such as fresh chicken and quails (Silva et al., 2001; Thiangtum et al., 2006). The prevalence of *T. gondii* infection in chicken was confirmed in many countries such as Australia, Ethiopia, and China. Even though there is no report in chicken in Thailand, the higher incidence was claimed (Yang et al., 2012; Chumpolbunchorn et al., 2013; Tilahun et al., 2013). Personal hygienic protocol and, classically, consumption of undercooked meat, particularly pork and lamb, have been ascribed to be the major risk factors for the acquisition of toxoplasmosis. Improved animal husbandry practices as well as increased awareness of the risks of undercooked meat have resulted in decreased prevalence of toxoplasmosis worldwide.

There are some evidences showing the possibility of co-infection of *T. gondii* with Feline Leukemia virus (FeLV) infection in captive wild felids. A previous study by Tangdujai and colleagues (2010) surveyed FeLV infection in smuggled felids in wildlife rehabilitation station at 2 wildlife breeding centers and found high prevalence of FeLV infection associated in their existed clinical signs. The coincidence with FeLV infection could induce both suppressive and neoplastic diseases of the feline lymphoreticular system and persistently viremic cats are known to be at increased risk of succumbing to diseases associated with FeLV. Toxoplasmosis is one of the common secondary diseases in retrovirus-infected cats, which coincides with the result of this study (Stuke et al., 2014).

This is the first report on the relation of *T. gondii* infection with strain identification in domestic cats and captive and free-range felids in Thailand. Better understanding of the genetic diversity will lead to better management, prevention and treatment of this disease in the valuable species of wild felids. This present study determined the coding sequences of the *T. gondii SAG4 and B1* genes and revealed relatively considerable sequence variability within this locus among *T. gondii* isolates from different hosts and geographical regions. The phylogenetic analysis showed that the *B1* gene could be used to distinguish between type I and type II, while the *SAG4* gene could be used to distinguish between type I and type III. More loci should be further analyzed to confirm the genotype, or another species characterization should be done to prove the genetic variation in *T. gondii* isolates in this type of animals in Thailand. Therefore, *SAG4* and *B1* might serve as novel genetic markers for population genetic studies of *T. gondii* isolates.

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บทคัดย่อ

ความชุกและการวิเคราะห์พันธุกรรมของเชื้อ *Toxoplasma gondii* ในแมวบ้าน สัตว์ป่าตระกูล

แมวในกรงเลี้ยง สัตว์ป่าตระกูลแมวในธรรมชาติ และสัตว์กลุ่มหนูที่เป็นเหยื่อ ในประเทศไทย

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โรคท็อกโซพลาสโมซิสหรือโรคไข้ขี้แมวเป็นโรคติดต่อจากสัตว์สู่คนในกลุ่มที่เกิดจากเชื้อปรสิตที่อยู่ในมนุษย์และสัตว์   เกิดจากการ
ติดเชื้อ *Toxoplasma gondii* รูปแบบของการติดเชื้อมีความแตกต่างกันในแต่ละพื้นที่ตามลักษณะทางภูมิศาสตร์ การศึกษานี้มีวัตถุประสงค์
เพื่อสำรวจอุบัติการณ์และจีโนไทป์ของการติดเชื้อ *T. Gondii* ในแมวบ้าน สัตว์ป่าตระกูลแมวในกรงเลี้ยงและในธรรมชาติ และสัตว์เลี้ยงลูกด้วย
นมขนาดเล็กที่เป็นเหยื่อ คือ หนู ทำการสำรวจในสัตว์ทั้ง 4 กลุ่มในประเทศไทยเป็นจำนวน 293 ตัวอย่าง และตรวจหาแอนติบอดีจำเพาะโดย
วิธี Indirect latex agglutination test (ILAT) และหาสารพันธุกรรมของยีน *glycerol-3-phosphate (B1)* และยีน *bradyzoite surface
antigen 4 (SAG4)* โดยวิธีปฏิกิริยาลูกโซ่โพลีเมอเรส (nested-PCR) จากการตรวจด้วยวิธี ILAT ในสัตว์ทุกกลุ่มทั้งหมดจำนวน 293 ตัวอย่าง
พบมีผลบวกร้อยละ 11.68 เมื่อแยกตามกลุ่มสัตว์พบผลบวกในแมวบ้านร้อยละ 3.48 (n=86) ในสัตว์ตระกูลแมวในกรงเลี้ยงร้อยละ 18.84
(n=138) ในเสือในธรรมชาติร้อยละ 14.28 (n=7) และในหนูชนิดที่เป็นเหยื่อตามธรรมชาติร้อยละ 6.67 (n=60) เมื่อนำตัวอย่างจากหนูที่ให้
ผลบวกโดยวิธี ILAT ได้แก่ เนื้อเยื่อจากตับ ไต สมอง และกล้ามเนื้อไปตรวจพันธุกรรมของเชื้อ *T. gondii* ด้วยวิธี nested-PCR พบว่าเนื้อเยื่อ
สมองและกล้ามเนื้อจากหนูนา (black rats) จำนวน 3 ตัวและอีก 1 ตัวอย่างจากเสือลายเมฆให้ผลการตรวจเป็นบวก เมื่อทำการเปรียบเทียบ
รหัสพันธุกรรมของยีน *B1* และ *SAG4* ของทั้ง 4 ตัวอย่างที่ตรวจพบนั้นด้วย ML Phylogenetic analysis พบว่าลำดับสารพันธุกรรมมีความ
เหมือนกับ *T. gondii* สายพันธุ์อ้างอิง RH type I ประมาณร้อยละ 99 และ 100 ตามลำดับ การตรวจพบอุบัติการณ์ของการติดเชื้อและการ
แยกสายพันธุ์ของเชื้อในครั้งนี้ในแมวบ้าน สัตว์ป่าตระกูลแมวในกรงเลี้ยงและที่อยู่ในธรรมชาติ และสัตว์กลุ่มหนูจะทำให้สามารถเข้าใจเกี่ยวกับ
สายพันธุ์ย่อยของเชื้อการก่อโรค การป้องกันรักษา และการจัดการที่ดีขึ้นเป็นลำดับต่อไป

คำสำคัญ: ท็อกโซพลาสมา กอนได แมวบ้าน สัตว์ป่าตระกูลแมว หนู การวิเคราะห์พันธุกรรม

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