EISSN: 2231-0916 doi: 10.14202/vetworld.2026.4609-4623 OPEN ACCESS

Veterinary World

RESEARCH ARTICLE

Molecular detection and multilocus genotyping of *Toxoplasma gondii* in archived stray-cat fecal DNA from central and western Thailand

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ABSTRACT

Background and Aim: *Toxoplasma gondii* is a globally important zoonotic protozoan, and felids serve as definitive hosts. Although molecular studies have demonstrated substantial genetic diversity of *T. gondii* in feline populations, information from Thailand remains limited, particularly for stray cats in central and western regions. This retrospective study aimed to determine the prevalence of *T. gondii* DNA and characterize multilocus genotype profiles in archived DNA extracted from stray-cat fecal samples collected in Nakhon Nayok and Kanchanaburi provinces, Thailand, in 2013.

Materials and Methods: A total of 120 archived fecal DNA samples were analyzed, comprising 80 samples from Nakhon Nayok and 40 from Kanchanaburi. Conventional polymerase chain reaction (PCR) targeting the *B1* gene was used for molecular detection. Positive amplicons were confirmed by Sanger sequencing and compared with GenBank reference sequences using Basic Local Alignment Search Tool. Sequence-confirmed samples were subsequently analyzed using multilocus nested PCR-restriction fragment length polymorphism (Mn-PCR-RFLP). Reference strains RH, PRU, and VEG represented clonal types I, II, and III, respectively. Differences in PCR positivity between provinces were assessed using Fisher's exact test.

Results: Thirteen of 120 samples (10.8%) were positive for *T. gondii* DNA, including 8/80 (10.0%) from Nakhon Nayok and 5/40 (12.5%) from Kanchanaburi; the difference was not significant (p = 0.758). B1 sequences showed 94.52%–98.70% identity with reference sequences. Multilocus genotyping was successful in four of 13 positive samples (30.8%). Two type I profiles and one type III profile were identified in Kanchanaburi, whereas one probable type II profile was identified in Nakhon Nayok. No mixed, atypical, or ToxoDB#9 profiles were detected among the four successfully characterized samples.

Conclusion: This study provides preliminary historical molecular evidence of classical *T. gondii* clonal-lineage profiles in stray-cat fecal DNA from central and western Thailand. However, the limited genotyping success prevents estimation of genotype frequencies or regional distribution. Detection of *T. gondii* DNA in feces should not be interpreted as evidence of viable oocysts, active shedding, infectivity, or current public health risk. Larger prospective studies using fresh samples, quantitative PCR, and higher-resolution genotyping are warranted.

Keywords: archived DNA, feline toxoplasmosis, molecular epidemiology, multilocus genotyping, stray cats, Thailand, *Toxoplasma gondii*, toxoplasmosis.

INTRODUCTION

Toxoplasma gondii is a zoonotic protozoan parasite with a broad host range and is an important cause of morbidity and mortality in domestic animals, wildlife, and humans [1, 2]. Infection with *T. gondii* poses particularly serious health risks to immunocompromised individuals and may be fatal in this population [3–5]. Humans and animals can acquire *T. gondii* by ingesting food or water contaminated with oocysts shed by felids, consuming tissue cysts in raw or undercooked meat, or through transplacental transmission [6–8]. Cats, as the definitive hosts of *T. gondii*, play a central role in toxoplasmosis epidemiology because they shed environmentally resistant oocysts in their feces, facilitating contamination of soil, water, and food sources [9–11]. Parasite virulence and the host immune response influence the clinical outcome of toxoplasmosis [12–14]. In addition, host characteristics,

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Received: 11-06-2026, **Accepted:** 15-09-2026, **Published online:** 17-10-2026
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How to cite: Udonsom R, Mahittikorn A, Hasan T, Nishikawa Y, Popruk S. Molecular detection and multilocus genotyping of *Toxoplasma gondii* in archived stray-cat fecal DNA from central and western Thailand. Vet World, 2026, 19(10): 4609–4623.
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parasite strain, infecting dose, and route of transmission may contribute to differences in disease pathogenesis and severity [14].

Genetic characterization of *T. gondii* is important for understanding parasite population diversity and its potential relationships with host responses, disease severity, epidemiology, and transmission dynamics [15, 16]. Genotyping approaches have identified 189 distinct *T. gondii* genotypes recorded in the *Toxoplasma* database [17]. Studies involving humans and animals have demonstrated substantial genetic diversity and geographic variation among *T. gondii* isolates. The global population structure of the parasite includes three major clonal lineages, types I, II, and III, as well as recombinant and atypical strains [18–25]. Type II is the genotype most frequently detected in human infections, whereas types II and III are commonly reported in animals [26, 27]. In feline populations, atypical genotypes have been frequently identified, with type II reported as the second most prevalent genotype [28, 29]. Recent molecular surveys in Iran, Romania, and the United States between 2022 and 2024 have further shown the circulation of both classical and atypical genotypes among stray and domestic cats, emphasizing the ongoing epidemiological importance of felids in *T. gondii* transmission [29–32].

In Thailand, molecular information on *T. gondii* genotypes in feline fecal samples remains limited. To date, only two previous studies have characterized *T. gondii* genotypes in cats. In Phayao province, northern Thailand, researchers identified type I in domestic cats using the GRA6 marker [33]. In Songkhla province, southern Thailand, researchers detected type I, type III, type II/III, recombinant, and atypical profiles in household cats using a five-marker polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) panel [34]. However, differences in geographic setting, cat population, and the number and type of genetic markers used limit direct comparison between these studies.

A substantial research gap remains regarding the molecular occurrence and multilocus genotype profiles of *T. gondii* in stray cats from central and western Thailand. In particular, published multilocus genotype data from feline fecal samples collected in Nakhon Nayok and Kanchanaburi provinces are scarce, limiting understanding of the historical genetic profiles present in these locations and preventing meaningful comparison with previously studied regions of Thailand. Available Thai studies have largely focused on northern and southern provinces and have used different genotyping approaches, leaving geographic and methodological gaps in the available evidence. Nakhon Nayok and Kanchanaburi were therefore of interest because stray-cat populations in these areas may live close to human communities and animal shelters, creating opportunities for environmental contamination with feline fecal material and potential parasite transmission. Nevertheless, because the present investigation used archived samples collected in 2013, its findings represent historical molecular baseline data and should not be interpreted as reflecting the current prevalence or genotype distribution of *T. gondii* in these provinces or in Thailand as a whole.

Therefore, this retrospective molecular study aimed to determine the prevalence of *T. gondii* DNA in archived DNA extracted from stray-cat fecal samples collected in Nakhon Nayok and Kanchanaburi provinces in 2013 using conventional polymerase chain reaction (PCR) targeting the *B1* gene. The authors also aimed to characterize the multilocus genotype profiles of sequence-confirmed *T. gondii*-positive samples using multilocus nested PCR-restriction fragment length polymorphism (Mn-PCR-RFLP) and to compare the identified clonal-lineage profiles with those previously reported in feline populations. The study aimed to provide preliminary historical molecular data from two previously underrepresented geographic areas rather than estimate contemporary genotype frequencies or nationwide genotype distribution.

MATERIALS AND METHODS

Ethical approval

This retrospective molecular study was conducted in accordance with institutional guidelines for animal research. The retrospective molecular analysis of residual archived DNA was reviewed and approved in 2026 by the Animal Care and Use Committee of the Faculty of Tropical Medicine, Mahidol University (FTM-ACUC 012/2026E). Biosafety approval was obtained from the Institutional Biosafety Committee (TM 2026-017). These approvals specifically covered the retrospective molecular analysis of archived DNA; no new animals were recruited, handled, restrained, or sampled for the present study. The original fecal specimens had been collected non-invasively from the ground immediately after defecation by observed stray cats, without direct animal handling or intervention. The original fecal specimens were collected in 2013 from the ground after defecation, with permission from the owner or caretaker of each shelter. Permission from the shelters did not constitute

institutional animal ethics approval. Therefore, the present investigation involved only laboratory analysis of residual DNA extracted from previously collected fecal specimens.

Study period and location

The retrospective molecular analysis of the archived DNA was conducted during late March and April 2026 at the Faculty of Tropical Medicine, Mahidol University, Thailand. The archived DNA analyzed in this retrospective study originated from fecal specimens collected from stray cats between July and September 2013 in two provinces of Thailand. Of the 120 archived DNA samples, 80 originated from cats housed at an animal refuge in Nakhon Nayok province, central Thailand (CN), and 40 originated from cats at shelter facilities in Kanchanaburi province, western Thailand (CK). Following ethics approval, the retrospective molecular analyses of the archived DNA were performed. Conventional PCR and preparation of PCR products for DNA sequencing were carried out at the Department of Protozoology, Faculty of Tropical Medicine, Mahidol University, Thailand. *T. gondii* multilocus nested PCR–RFLP genotyping was subsequently conducted at the National Research Center for Protozoan Diseases, Obihiro University of Agriculture and Veterinary Medicine, Japan.

Study design

No prospective sample size calculation was performed. The study included all 120 archived DNA samples available for retrospective analysis. This retrospective molecular study included 120 archived DNA samples extracted from fecal specimens collected opportunistically from stray cats housed at two animal shelters: one in Nakhon Nayok province and one in Kanchanaburi province. Sampling at the time of the original collection was based on convenience sampling of available animals. Only one fecal sample was collected from each cat observed defecating; therefore, each sample represented a different observed animal. Immediately after observing a cat defecate, collectors obtained one fecal specimen and applied a temporary-colored mark to the cat to indicate that it had been sampled. Cats appeared healthy based on observations at the time of sample collection. Information on age, sex, neuter status, and individual clinical history was unavailable. Archived samples were eligible for the present analysis when the sample identification and collection location could be verified from laboratory records and sufficient residual DNA was available for molecular analysis. No predetermined DNA concentration or spectrophotometric purity threshold was applied.

Fecal sample collection and DNA extraction

Fecal specimens were collected from the ground immediately after defecation, transported under refrigerated conditions at 2–6°C, and stored at −20°C until DNA extraction. Genomic DNA was extracted using the PSP® Spin Stool DNA Kit (Stratec Molecular, Berlin, Germany) according to the manufacturer's instructions. The extraction procedure included a pre-lysis step with Zirconia Beads II and treatment with InviAdsorb to reduce PCR inhibitors. DNA was eluted in 100 µL of elution buffer and stored at −20°C until subsequent molecular analyses. Records of aliquoting and the number of freeze–thaw cycles were unavailable.

***T. gondii* DNA detection by PCR**

Conventional PCR targeting the *B1* gene was performed to detect *T. gondii* DNA using the following primers: *B1*_forward, 5′-AACGGGCGAGTAGCACCTGAGGAGA-3′, and *B1*_reverse, 5′-TGGGTCTACGTCGATGGCATGACAAC-3′ [35]. PCR was performed using a Vapo.protect Mastercycler® Pro thermocycler (Eppendorf, Hamburg, Germany) in a final reaction volume of 20 µL containing 1× $MgCl_2$-free PCR buffer, 0.25 mM of each dNTP, 2 mM $MgCl_2$, 0.25 µM of each primer, 0.5 U of Taq DNA polymerase (Thermo Fisher Scientific, Waltham, MA, USA), and 2 µL of genomic DNA template. DNA extracted from *T. gondii* RH-strain tachyzoites and nuclease-free water served as positive and negative PCR controls, respectively, and were included in every experiment.

To prepare the positive controls and assess the limit of detection (LOD), *T. gondii* RH-strain tachyzoites obtained from cell culture were counted and prepared at concentrations ranging from 1 to 1×10^5 tachyzoites in 100 µL of phosphate-buffered saline before DNA extraction. *T. gondii* RH-strain tachyzoites used to prepare the *B1* PCR positive control were grown in cell culture at the Department of Protozoology, Faculty of Tropical Medicine, Mahidol University, Thailand. The strain has been maintained at the department for more than 40 years. The LOD of the *B1* PCR assay was evaluated using DNA extracted from serially diluted tachyzoite preparations. Three independent dilution series were prepared. In each experiment, DNA was extracted separately from every dilution, and the complete series was tested in duplicate *B1* PCR reactions. The lowest input that consistently produced amplification in duplicate reactions was DNA extracted from a preparation containing one tachyzoite before DNA extraction. This experiment assessed analytical performance using tachyzoite

preparations only and did not incorporate an oocyst wall or fecal matrix. Therefore, this LOD should not be extrapolated to the analytical or diagnostic sensitivity of the assay for detecting oocysts in fecal specimens, in which DNA extraction efficiency and PCR inhibitors may influence assay sensitivity.

PCR amplification consisted of initial denaturation at 95°C for 5 min, followed by 40 cycles of denaturation at 95°C for 15 s, annealing at 60°C for 15 s, and extension at 72°C for 1 min, with a final extension at 72°C for 7 min. The 115-bp PCR products were analyzed by electrophoresis on 1.8% agarose gels containing RedSafe™ Nucleic Acid Staining Solution (IntRON Biotechnology, Seongnam, South Korea) and visualized under ultraviolet illumination. PCR amplification was independently repeated for all positive samples, and consistent results were obtained.

Sequencing and sequence analysis

All PCR-positive products were subjected to Sanger sequencing using primers targeting the *B1* gene on an ABI 3730xl DNA sequencer (Applied Biosystems, Foster City, CA, USA). The *B1* amplicons were sequenced in both forward and reverse directions, except for sample CK35, for which only forward-sequence data were available because reverse-sequence data could not be obtained. The generated sequences were aligned and compared with reference sequences available in the GenBank database using the Basic Local Alignment Search Tool (BLAST).

Genotyping of *T. gondii*

Samples confirmed positive for *T. gondii* by DNA sequencing were subjected to genotyping using 11 genetic marker systems: SAG1, combined 5′- and 3′-SAG2, alt.SAG2, SAG3, GRA6, BTUB, C22-8, C29-2, L358, PK1, and Apico, as previously described [36, 37]. The 5′- and 3′-SAG2 regions were amplified separately but interpreted jointly and were therefore considered a single SAG2 marker system. Of the 11 marker systems initially examined, eight, SAG1, combined 5′- and 3′-SAG2, alt.SAG2, SAG3, GRA6, C22-8, C29-2, and L358, generated reproducible and interpretable profiles in the RH, PRU, and VEG reference strains and were retained for genotype interpretation. BTUB, PK1, and Apico did not amplify reproducibly in the reference strains under the experimental conditions and were therefore excluded from genotype interpretation. Definitive clonal-lineage assignment required reproducible results from at least six of the eight retained marker systems, with all successfully typed discriminatory markers matching a single reference lineage.

Genotype identification was performed using multilocus nested PCR-restriction fragment length polymorphism (Mn-PCR-RFLP) according to previously described protocols [38, 39]. Briefly, first-round multiplex PCR was performed using external primers for each marker, except 5′-SAG2, for which no external primer was used because its DNA sequence is covered by alt.SAG2. The 25-µL multiplex PCR mixture contained 2.5 µL of 10× PCR buffer containing 15 mM $MgCl_2$, 0.2 µL of AmpliTaq DNA polymerase (5 U/µL), 2.5 µL of dNTP mixture (2 mM), 0.15 µL of each external forward and reverse primer (50 µM), 2 µL of DNA template, and 17.5 µL of nuclease-free water. Thermal cycling consisted of initial denaturation at 95°C for 4 min, followed by 30 cycles at 94°C for 30 s, 55°C for 1 min, and 72°C for 2 min, with a final extension at 72°C for 5 min. Genomic DNA preparations from the *T. gondii* RH, PRU, and VEG reference strains at concentrations of 27.0, 21.3, and 24.0 ng/µL, respectively, served as positive controls representing types I, II, and III. Nuclease-free water served as the negative control.

The 25 µL first-round multiplex PCR product was mixed with 25 µL nuclease-free water to obtain a twofold (1:2) dilution. Second-round PCR was performed separately for each marker in a final volume of 25 µL containing 2.5 µL of 10× PCR buffer with 15 mM $MgCl_2$, 2.5 µL of dNTP mixture (2 mM), 0.3 µL of each internal primer (50 µM), 0.2 µL of AmpliTaq DNA polymerase (5 U/µL), 2 µL of diluted first-round PCR product, and 17.2 µL of nuclease-free water.

To determine RFLP patterns, 5 µL of each second-round PCR product was digested with the appropriate restriction enzyme (New England Biolabs, Ipswich, MA, USA) for each marker and subsequently analyzed by agarose gel electrophoresis. A sample was considered successfully genotyped when reproducible PCR-RFLP patterns were obtained from multiple independent genetic markers, permitting comparison with the reference clonal lineages. Because SAG1 alone does not reliably discriminate between type II and type III lineages, genotype determination was based on concordant results across multiple discriminatory loci rather than on SAG1 alone. Ambiguous findings at an individual marker were interpreted in the context of the overall multilocus genotype profile and were not used independently for lineage assignment.

A genotype was assigned only when concordant profiles from multiple discriminatory markers matched the corresponding reference strain. Each successfully amplified marker was independently examined twice in separate PCR-RFLP experiments and was included in genotype assignment only when concordant amplification

and restriction-fragment patterns were obtained in both experiments. Negative controls containing nuclease-free water were included in every Mn-PCR-RFLP run. Primer sequences, expected amplicon sizes, restriction enzymes, digestion conditions, and reference-strain RFLP patterns for all 11 marker systems are provided in Supplementary Table 1.

Statistical analysis

PCR positivity was reported with 95% confidence intervals calculated using the Wilson score method (Statistics Fundamentals Wilson Score Calculator, https://statisticsfundamentals.com/calculators/wilson-score/). Differences between provinces were assessed using a two-sided Fisher's exact test (MedCalc online calculator, https://www.medcalc.org/en/calc/fisher.php). Statistical significance was set at $p < 0.05$.

RESULTS

Detection and sequence confirmation of *T. gondii* DNA

Fecal samples were obtained from stray cats in Nakhon Nayok and Kanchanaburi provinces. Information on age, sex, neuter status, and individual clinical history was unavailable because the sampled animals were stray cats of unknown origin and history.

T. gondii DNA was detected in 8 of 80 fecal samples from Nakhon Nayok province (10.0%; 95% CI = 5.2–18.5) and 5 of 40 samples from Kanchanaburi province (12.5%; 95% CI = 5.5–26.1). Overall, 13 of 120 samples (10.8%; 95% CI = 6.4–17.7) were positive. No statistically significant difference in PCR positivity was observed between the two provinces (p = 0.758; Table 1).

Table 1: Polymerase chain reaction (PCR) positivity of *Toxoplasma gondii* DNA in fecal samples from stray cats in Nakhon Nayok and Kanchanaburi provinces detected by conventional PCR targeting the *B1* gene.

Location	No. of stray cats	No. of positive samples (%)	95% CI	p-value
Nakhon Nayok	80	8 (10.0)	5.2–18.5	0.758
Kanchanaburi	40	5 (12.5)	5.5–26.1	
Total	120	13 (10.8)	6.4–17.7	

CI = Confidence interval. The difference in PCR positivity between Nakhon Nayok and Kanchanaburi provinces was evaluated using a two-sided Fisher's exact test.

The 115-bp PCR amplification products are shown in Figure 1. Four positive cat fecal samples are shown in lanes 8, 11, 16, and 17. A faint amplification band was observed in lane 8 compared with the other positive samples and the positive control. Sequencing of the *B1* gene demonstrated 94.52%–98.70% sequence identity between the 13 PCR-positive samples and *T. gondii* reference sequences available in GenBank. The percentage identity, query coverage, and closest GenBank accession number for each sample are provided in Supplementary Table 2.

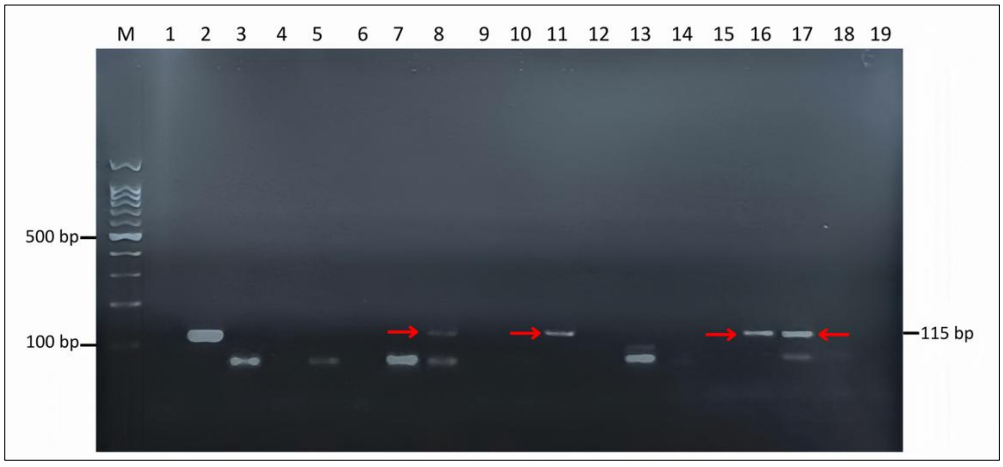


Figure 1: PCR amplification of the *Toxoplasma gondii B1* gene and analysis of the 115-bp amplicons by 1.8% agarose gel electrophoresis. Lane M = 100-bp DNA ladder; lane 1 = negative control; lane 2 = positive control (115 bp); lanes 3–19 = cat fecal samples. PCR = Polymerase chain reaction; DNA = Deoxyribonucleic acid.

Mn-PCR-RFLP genotyping

Multilocus nested PCR-restriction fragment length polymorphism (Mn-PCR-RFLP) genotyping was successful for 4 of the 13 *T. gondii*-positive samples (30.8%). Sample CK31 yielded interpretable results for eight marker

systems: SAG1, combined 5′- and 3′-SAG2, alt.SAG2, SAG3, GRA6, C22-8, C29-2, and L358. Samples CK34 and CK35 were each characterized using six marker systems: alt.SAG2, SAG3, GRA6, C22-8, C29-2, and L358. Sample CN36 was characterized using four marker systems: SAG1, combined 5′- and 3′-SAG2, alt.SAG2, and SAG3 (Table 2).

Based on concordant profiles obtained for the combined 5′- and 3′-SAG2, alt.SAG2, and SAG3 markers, sample CN36 was provisionally classified as a probable type II profile. SAG1 provided an additional result but was not independently discriminatory between types II and III. The remaining marker systems could not be evaluated for CN36 because insufficient archived DNA was available. Overall, two type I profiles and one type III profile were identified among samples from Kanchanaburi province, whereas one probable type II profile was identified in Nakhon Nayok province. No mixed, atypical, or ToxoDB#9 profiles were detected among the four successfully characterized samples.

Marker amplification and PCR-RFLP patterns

Amplification success varied by marker and sample. Sample CK31 yielded amplification for SAG1, combined 5′- and 3′-SAG2, alt.SAG2, SAG3, GRA6, C22-8, C29-2, and L358. Samples CK34 and CK35 yielded identical sets of successfully amplified markers comprising alt.SAG2, SAG3, GRA6, C22-8, C29-2, and L358. Sample CN36 yielded amplification for SAG1, combined 5′- and 3′-SAG2, alt.SAG2, and SAG3; the remaining markers could not be examined because insufficient archived DNA remained. Accordingly, the genotype assignment for CN36 was considered provisional and was based on concordant results from the successfully amplified loci (Table 2).

The Mn-PCR-RFLP patterns are presented in Figure 2. Most successfully amplified markers produced distinct bands. Faint amplification bands were observed for the 5′-SAG2 marker in lanes 3 and 4, corresponding to the VEG type III control and sample CK31, respectively. Faint bands were also observed for the C22-8 marker in cat samples loaded in lanes 4–6 and for the C29-2 marker in sample CK31 (lane 4). No evidence of mixed *T. gondii* infection was detected among the successfully genotyped samples. Negative controls containing nuclease-free water remained negative in every Mn-PCR-RFLP run, with no detectable amplification products.

Table 2: Summary of Mn-PCR-RFLP genotyping outcomes for *Toxoplasma gondii* DNA-positive fecal samples from stray cats.

Province	Case ID (N = 13)	Markers amplified	Genotyping result
Central region, Nakhon Nayok	CN22	Not successfully amplified	Genotype not determined
	CN24	Not successfully amplified	Genotype not determined
	CN30	Not successfully amplified	Genotype not determined
	CN33	Not successfully amplified	Genotype not determined
	CN36	4 markers: SAG1, combined 5′- and 3′-SAG2, alt.SAG2, and SAG3; remaining loci not tested because of insufficient DNA	Probable type II
	CN41	Not successfully amplified	Genotype not determined
	CN43	Not successfully amplified	Genotype not determined
	CN55	Not successfully amplified	Genotype not determined
Western region, Kanchanaburi	CK13	Not successfully amplified	Genotype not determined
	CK28	Not successfully amplified	Genotype not determined
	CK31	8 markers: SAG1, combined 5′- and 3′-SAG2, alt.SAG2, SAG3, GRA6, C22-8, C29-2, and L358	Type I
	CK34	6 markers: alt.SAG2, SAG3, GRA6, C22-8, C29-2, and L358	Type III
	CK35	6 markers: alt.SAG2, SAG3, GRA6, C22-8, C29-2, and L358	Type I

CK = Cat fecal samples collected in Kanchanaburi province; CN = Cat fecal samples collected in Nakhon Nayok province; Mn-PCR-RFLP = Multilocus nested polymerase chain reaction-restriction fragment length polymorphism.

DISCUSSION

Molecular detection of *T. gondii* in stray-cat fecal samples

Molecular investigation of feline populations can provide valuable insights into the epidemiology and genetic diversity of *T. gondii* [17, 30, 40]. However, detection of *T. gondii* DNA in feline feces should be interpreted cautiously because molecular positivity does not necessarily confirm the presence of intact or viable oocysts or active oocyst shedding [41–43].

In the present study, *T. gondii* DNA was detected in 10.8% of stray-cat fecal samples. This prevalence was lower than the 37.1% reported in domestic cats from Phayao province, northern Thailand [33], but higher than the 6.7% reported in household cats from Songkhla province, southern Thailand [44], and the 2.5% reported in stray cats from Bangkok, Thailand [43]. A comparable prevalence of 8.5% has been reported in Malaysia [45], whereas higher prevalence rates of 15.2%–18.2% have been reported in Iran [31] and a lower prevalence of 4.7%

in Korea [46]. This study provides the report of *T. gondii* DNA detection, along with preliminary multilocus genotype profiles, from stray-cat fecal samples collected in Nakhon Nayok and Kanchanaburi provinces. Differences in molecular prevalence among studies may be associated with variation in feline populations, management practices, environmental conditions, sample size, geographic setting, sample characteristics, and molecular detection methods. Nevertheless, the convenience sampling design and small number of DNA-positive samples in the present study do not support inference of geographic differences in prevalence.

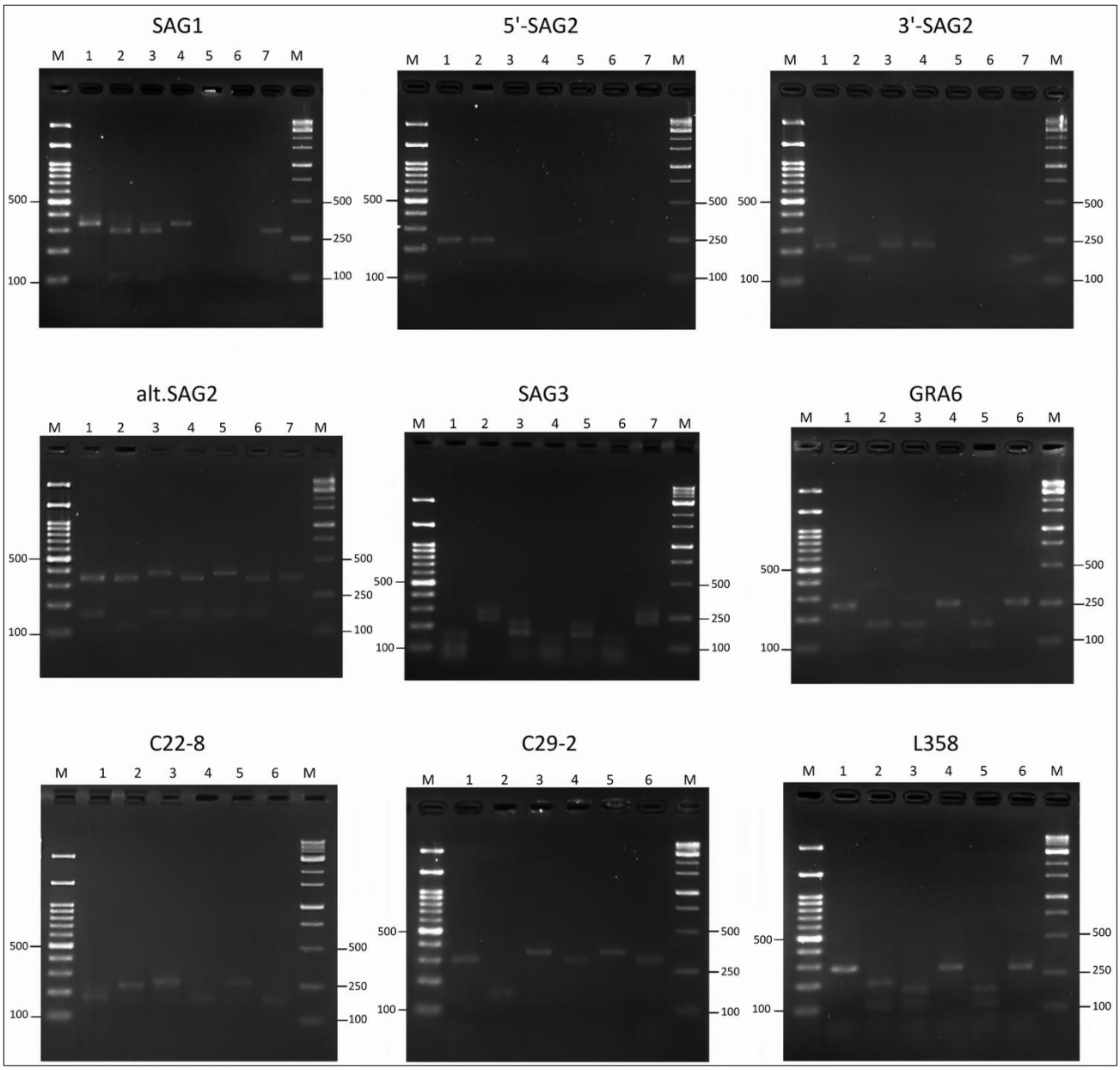


Figure 2: Multilocus nested polymerase chain reaction-restriction fragment length polymorphism (Mn-PCR-RFLP) characterization of *Toxoplasma gondii* DNA-positive fecal samples. Lane M = 100-bp DNA ladder (left) or 1-kb DNA ladder (right); lane 1 = RH reference strain (type I); lane 2 = PRU reference strain (type II); lane 3 = VEG reference strain (type III); lanes 4–7 = cat fecal samples CK31, CK34, CK35, and CN36, respectively. The residual DNA volume for sample CN36 was insufficient for analysis of GRA6, C22-8, C29-2, and L358. Mn-PCR-RFLP = Multilocus nested polymerase chain reaction-restriction fragment length polymorphism; DNA = Deoxyribonucleic acid; CK = Cat fecal sample collected in Kanchanaburi province; CN = Cat fecal sample collected in Nakhon Nayok province.

Multilocus genotype profiles in Thailand

The present study further characterized *T. gondii* genotype profiles in stray-cat fecal samples from the two study areas. Mn-PCR-RFLP identified two type I profiles and one type III profile among successfully genotyped samples from Kanchanaburi province and one probable type II profile in Nakhon Nayok province.

Only two previous studies in Thailand have reported *T. gondii* genotypes in feline fecal samples. Suwancharoen *et al.* [33] identified type I in DNA-positive samples from domestic cats in Phayao province using

the GRA6 marker, whereas Chemoh *et al.* [34] reported type I, type III, type II/III, recombinant, and atypical profiles in household cats from Songkhla province using a five-marker PCR-RFLP panel. Differences in the feline populations, geographic settings, and genotyping approaches used among these studies should be considered when comparing their findings.

The multilocus profiles identified in the present study were considered in the context of *T. gondii* genotypes previously reported in feline hosts worldwide (Table 3) [29–34, 46–74]. Published findings vary substantially according to geographic region, host population, sample type, and genotyping method. The present findings extend the available historical molecular data to Nakhon Nayok and Kanchanaburi provinces. However, only four of the 13 DNA-positive samples yielded interpretable multilocus profiles; therefore, the present data cannot be used to estimate genotype frequencies or establish differences in genotype distribution among regions of Thailand.

Table 3: Overview of *Toxoplasma gondii* genotypes reported in feline hosts worldwide, grouped by continent and geographic region.

Country/region	Feline host	Sample type	Positive samples or isolates/No. examined	Positivity rate (%)	Technique	Genotype(s)	Reference
Asia							
Thailand, Nakhon Nayok	Stray cats	Feces	8/80	10.0	Mn-PCR-RFLP	Probable type II	Present study
Thailand, Kanchanaburi	Stray cats	Feces	5/40	12.5	Mn-PCR-RFLP	I, III	Present study
Thailand, Phayao	Domestic cats	Feces	46/124	37.1	GRA6-locus PCR-RFLP	I	[33]
Thailand, Songkhla	Pet cats	Feces	8/13	61.5	Mn-PCR-RFLP	I, III, II or III, recombinant, atypical	[34]
Iran, Khorramabad	Stray cats	Feces	13/200	6.5	GRA6-locus PCR-RFLP	III	[30]
Iran, Tehran	Stray cats; house cats	Feces	20/132; 6/33	15.2; 18.2	Mn-PCR-RFLP	I, II, III	[31]
Iran, Mashhad	Stray cats	Feces; brain	8/175; 1/31	4.6; 3.2	Bioassay, Mn-PCR-RFLP	II	[47]
Japan, Okinawa	Stray cats	Brain, heart	9/201	4.5	Bioassay, GRA6-locus PCR-RFLP	I, II	[48]
China, Jiangsu	Stray cats	Brain, heart, lung, muscle	11/64	17.2	Mn-PCR-RFLP	ToxoDB#9, ToxoDB#10, ToxoDB#89, ToxoDB#213, new genotypes 1 and 2	[49]
China, Henan	Captive felids	Heart	1/40	2.5	Bioassay, Mn-PCR-RFLP	ToxoDB#9	[50]
China, Yunnan	Cats	Feces	5/115	4.3	Bioassay, DNA sequencing-based GRA6 and SAG2	II	[51]
China, Yunnan	Stray cats	Brain, tongue, heart, liver	44/175	25.1	Mn-PCR-RFLP	II, ToxoDB#3, ToxoDB#9, ToxoDB#20, ToxoDB#225	[52]
China, Zhejiang/Henan/Beijing	Cats	Tongue, heart, feces	8/42; 1/318	19.0; 0.3	Bioassay, *in vitro* culture, Mn-PCR-RFLP	ToxoDB#2, ToxoDB#9, ToxoDB#17, ToxoDB#1	[53]
China, Guizhou	Stray cats	Brain	10/19	52.6	Bioassay, Mn-PCR-RFLP	Chinese 1	[54]
China, Beijing	Stray cats	Brain, tongue, heart	11/23	47.8	Bioassay, Mn-PCR-RFLP	ToxoDB#9	[55]
China, Anhui/Hubei/Shanxi/Guangdong	Cats	Brain, tongue, heart	14/89	15.7	Bioassay, Mn-PCR-RFLP	Chinese 1, Chinese 2	[56]

Country/region	Feline host	Sample type	Positive samples or isolates/No. examined	Positivity rate (%)	Technique	Genotype(s)	Reference
Pakistan, Punjab	Cats	Feces	5/400	1.25	DNA sequencing-based SAG2	Atypical type (AF249696)	[57]
Korea, Seoul	Stray cats	Feces	14/300	4.7	Bioassay, DNA sequencing-based SAG5D and SAG5E	II	[46]
Korea, Gyeonggi-do	Stray cats	Blood	13/23	56.5	GRA6-locus PCR-RFLP	I	[58]
Turkey, Izmir	Stray cats	Brain, heart	22/100	22.0	Microsatellites	II, III, Africa 1	[59]
Qatar/United Arab Emirates	Sand cats	Brain, tongue, heart	1/27; 3/57	3.7; 5.3	Bioassay, Mn-PCR-RFLP	II, atypical	[60]
Africa							
Egypt, Giza	Feral cats	Brain, tongue, heart	115/135	85.2	Mn-PCR-RFLP	II, III, atypical	[61]
Ethiopia, Addis Ababa	Feral cats	Heart; feces	26/48; 1/48	54.1; 2.1	Mn-PCR-RFLP	ToxoDB#1, ToxoDB#2, ToxoDB#3, ToxoDB#20	[62]
Algeria	Stray cats	Heart	87/96	90.6	Microsatellites	II, II variant	[63]
Australia							
Australia, Sydney	Domestic cats	Brain, liver, lung, skeletal muscle	11/17	64.7	Mn-PCR-RFLP	ToxoDB#3, type II-like	[64]
Europe							
Romania, Cluj/Alba/Bistriţa Năsăud	Domestic cats	Feces; heart	2/31; 7/27	6.4; 25.9	Bioassay, Mn-PCR-RFLP	ToxoDB#1 or ToxoDB#3; ToxoDB#2	[29]
Portugal, Lisbon	Stray cats	Brain	82/164	50.0	Bioassay, microsatellites	II, recombinant strain	[65]
Switzerland, Bern/Olten	Pet cats	Feces	1/252	0.4	Mn-PCR-RFLP	II	[66]
Germany	Cats	Feces	68/18,259	0.4	Bioassay, Mn-PCR-RFLP	II, III, atypical mixed type	[67]
Spain, La Rioja	Farm, stray, and house cats	Brain	47/592	7.9	Bioassay, SAG2-locus PCR-RFLP	I, II	[68]
North America							
United States, central coastal California	Feral cats	Feces	94/362	25.9	B1-locus PCR-RFLP, multilocus sequence typing	I, II/III, type X or X variants, atypical	[32]
Mexico, Colima	Cats	Brain	8/48	16.6	Bioassay, Mn-PCR-RFLP	III, ToxoDB#28	[69]
West Indies, St. Kitts	Feral cats	Brain, heart, tongue, feces	7/96	7.3	Bioassay, Mn-PCR-RFLP	II, III, two unique types	[70]
South America							
Brazil, Pernambuco	Feral cats	Brain, heart, diaphragm	2/31	6.4	Bioassay, Mn-PCR-RFLP	Atypical (ToxoDB#146)	[71]
Brazil, Rio Grande do Sul	Wild felids	Brain, liver, lung, skeletal muscle, diaphragm	31/90	34.4	Mn-PCR-RFLP	Lw#31Tn, Py#21Sm, Py#56Br, atypical, recombinant	[72]
Brazil, São Paulo	Stray cats	Brain, tongue, heart, feces	47/237; 3/237	19.8; 1.3	Bioassay, SAG2-locus PCR-RFLP	I, III, mixed I and III	[73]
Colombia, Armenia	Domestic cats	Feces	25/140	17.8	DNA sequencing-based ROP18	Archetype I Brazilian, Chinese strain	[74]

Mn-PCR-RFLP = Multilocus nested polymerase chain reaction-restriction fragment length polymorphism; PCR-RFLP = Polymerase chain reaction-restriction fragment length polymorphism.

Geographic diversity of *T. gondii* genotypes

Globally, types II and III are among the most frequently reported clonal lineages in animal toxoplasmosis [26, 27], whereas atypical genotypes have also been documented in feline hosts. Several studies from China have identified ToxoDB#9 (Chinese 1) among the genotypes circulating in cats (Table 3). In contrast, only classical clonal-lineage profiles were identified among the four successfully characterized samples in the present study. No

atypical, mixed, or ToxoDB#9 profiles were detected. However, nine of the 13 DNA-positive samples did not yield interpretable multilocus profiles, and the number of successfully genotyped samples was small. Consequently, atypical, recombinant, mixed, or other variants may have remained undetected, and the present findings cannot rule out their presence in the wider feline population.

Studies from other Asian regions further demonstrate geographic heterogeneity in feline *T. gondii* genotypes. Types I and II have been reported in cats from Japan and Korea, whereas studies from Iran have identified multiple clonal lineages, including types I, II, and III [30, 31, 46–48, 58]. In Turkey, stray cats have been reported to carry types II and III and the atypical Africa 1 genotype [59]. Collectively, these observations illustrate considerable variation in the population structure of *T. gondii* among feline populations. Nevertheless, direct comparison with the present findings should be undertaken cautiously because studies differ substantially in sample type, host population, molecular detection approach, and genotyping resolution.

In Europe, type II has been frequently reported in feline populations, including studies from Portugal, Switzerland, Germany, and Spain [65–68]. The probable type II profile identified in Nakhon Nayok is therefore compatible with a lineage reported in feline populations elsewhere; however, this finding should not be interpreted as evidence of epidemiological similarity between geographically distinct populations. The type I and III profiles detected in Kanchanaburi are also among the profiles previously reported in southern Thailand [34]. Mixed *T. gondii* genotypes have been reported in individual cats in Iran and Brazil [31, 73], while studies from several other countries have documented multiple genotypes among their sampled feline populations (Table 3).

Epidemiological and public health implications

Genetic differences among *T. gondii* strains have been associated with variation in parasite virulence and disease outcomes. Type I strains, in particular, have been associated with high virulence in experimental models and severe outcomes in some human infections. However, the present study identified type I DNA profiles in archived feline fecal DNA and did not assess viable oocysts, active oocyst shedding, infectivity, environmental exposure, or human infection. Therefore, detection of type I profiles in stray-cat fecal samples from the study areas cannot be interpreted as direct evidence of increased public health risk.

Nevertheless, the detection of *T. gondii* DNA and multiple classical clonal-lineage profiles in archived stray-cat fecal DNA demonstrates the historical molecular presence of the parasite in the sampled feline populations. These findings support the value of continued molecular surveillance using contemporary samples and approaches capable of distinguishing DNA detection from viable and potentially infectious oocysts. Such investigations would provide a stronger basis for assessing the epidemiological significance of feline-associated environmental contamination.

Study limitations and future perspectives

Several limitations should be considered when interpreting these findings. First, only four of the 13 *T. gondii* DNA-positive samples were successfully characterized by multilocus genotyping, preventing reliable estimation of genotype frequencies or regional distribution. The lower success of multilocus genotyping relative to *B1* detection may reflect low parasite DNA abundance, residual fecal PCR inhibitors, DNA degradation during prolonged storage, and the lower analytical sensitivity of some single-copy genotyping markers compared with the multicopy *B1* target.

Second, the study used residual DNA extracted from fecal specimens collected in 2013. The original fecal material was unavailable, precluding repeat DNA extraction, oocyst concentration, microscopic confirmation, and assessment of oocyst viability. Long-term storage may also have resulted in DNA fragmentation, and the number of freeze–thaw cycles experienced by the archived DNA could not be reconstructed.

Third, conventional *B1* PCR did not provide quantitative information on parasite DNA. Quantitative PCR was not performed because of the limited volume of residual archived DNA; therefore, parasite DNA burden could not be examined in relation to multilocus genotyping success. Furthermore, three initially evaluated marker systems, BTUB, PK1, and Apico, were excluded from genotype interpretation because they did not amplify reproducibly in the reference strains under the experimental conditions. Consequently, atypical, recombinant, mixed, or other variants may have remained undetected, particularly among the nine DNA-positive samples without interpretable multilocus profiles.

Fourth, *B1* PCR detects *T. gondii* DNA but does not establish the presence of intact or viable oocysts. Parasite DNA detected in feces may originate from oocysts, ingested infected material, residual non-viable DNA, or environmental contamination of fecal material after deposition. Therefore, PCR positivity in the present study

should not be interpreted as direct evidence of active oocyst shedding or infectivity.

Finally, the convenience sampling design and small number of successfully genotyped samples limit the generalizability of the findings. The archived samples nevertheless provide a historical molecular baseline for the two sampled provinces and may facilitate comparison with future investigations using freshly collected specimens. Future studies should incorporate larger, systematically selected feline populations, contemporary sampling, quantitative molecular detection, approaches to assess oocyst viability, and higher-resolution multilocus or sequence-based genotyping. Such studies would help determine whether the historical profiles identified here persist in contemporary feline populations and provide more representative information on *T. gondii* genetic diversity in Thailand.

CONCLUSION

This retrospective molecular study provides preliminary historical evidence of *T. gondii* DNA and multilocus genotype profiles in archived stray-cat fecal DNA from Nakhon Nayok and Kanchanaburi provinces, Thailand. *T. gondii* DNA was detected in 13 of 120 samples (10.8%), with comparable PCR positivity in Nakhon Nayok (10.0%) and Kanchanaburi (12.5%; $p = 0.758$). Among the 13 DNA-positive samples, four (30.8%) yielded interpretable multilocus profiles by Mn-PCR-RFLP: two type I profiles and one type III profile from Kanchanaburi, and one probable type II profile from Nakhon Nayok. No mixed, atypical, or ToxoDB#9 profiles were identified among these four successfully characterized samples; however, their absence from the wider feline population cannot be inferred because of the limited genotyping success.

A strength of this study is the combined use of *B1*-targeted PCR, sequence confirmation, and multilocus PCR-RFLP with reference strains and repeated marker testing, providing complementary molecular evidence for parasite detection and preliminary lineage characterization. In addition, analysis of archived samples provides a historical molecular baseline for two Thai provinces with limited feline multilocus genotype information. From a practical perspective, these findings support the value of including stray-cat populations in molecular surveillance of *T. gondii* and provide baseline data against which contemporary surveys may be compared. Nevertheless, detection of *T. gondii* DNA in feces does not demonstrate the presence of intact or viable oocysts, active shedding, infectivity, or direct public health risk.

The findings should therefore be interpreted within the constraints of convenience sampling, the small number of successfully genotyped samples, prolonged storage of residual DNA, and the inability to assess parasite burden or oocyst viability. Future prospective studies using freshly collected samples, larger and systematically selected feline populations, quantitative molecular assays, viability assessment, and higher-resolution multilocus or sequence-based genotyping are warranted. Such studies could determine whether the historical clonal-lineage profiles identified here persist in contemporary feline populations and provide more representative evidence of *T. gondii* genetic diversity and its epidemiological significance in Thailand.

DATA AVAILABILITY

The sample-level B1 sequence characteristics, BLASTn results, and Mn-PCR-RFLP outcomes generated in this study are provided in the Supplementary Material. The B1 sequences generated in this study were not deposited in GenBank. The supplementary data are available from the corresponding author upon reasonable request.

GENERATIVE AI DECLARATION

The authors declare that no generative artificial intelligence (AI) tools were used in the preparation, writing, editing, or revision of this manuscript.

AUTHORS' CONTRIBUTIONS

RU: Conceptualization, formal analysis, investigation, writing—original draft preparation, review, and editing. AM: Methodology and formal analysis. TH: Methodology. YN: Conceptualization, formal analysis, resources, project administration, review and editing of the manuscript, and funding acquisition. SP: Conceptualization, formal analysis, investigation, resources, project administration, writing—original draft preparation, review, and editing. All authors have read and approved the final version of the manuscript.

ACKNOWLEDGMENTS

This study was partially financially supported by the Faculty of Tropical Medicine, Mahidol University, and

the National Research Center for Protozoan Diseases, Obihiro University of Agriculture and Veterinary Medicine, Japan and the JSPS KAKENHI Grants (24H02292).

COMPETING INTERESTS

The authors declare that they have no competing interests.

PUBLISHER'S NOTE

Veterinary World remains neutral with regard to jurisdictional claims in the published institutional affiliations.

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