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FULL PAPER

Parasitology

Molecular characterization of the ribosomal DNA unit of *Sarcocystis singaporensis*, *Sarcocystis zamani* and *Sarcocystis zuoi* from rodents in Thailand

Vichan WATTHANAKAIWAN[1,2], Manakorn SUKMAK[3]*, Kriengsak HAMARIT[2], Nongnid KAOLIM[3], Worawidh WAJJWALKU[1,3] and Yuttamol MUANGKRAM[4]

[1]Interdisciplinary Graduate Program in Genetic Engineering, The Graduate School, Kasetsart University, Bangkok, 10900, Thailand
[2]Entomology & Zoology Research Group, Plant Protection Research & Development Office, Department of Agriculture, Chatuchuk, Bangkok, 10900, Thailand
[3]Faculty of Veterinary Medicine, Kasetsart University, Kamphaeng Sean Campus, Nakhon Pathom, 73140, Thailand
[4]Graduate School of Life Sciences, Ritsumeikan University, Kusatsu, Shiga 525–8577, Japan

ABSTRACT. *Sarcocystis* species are heteroxenous cyst-forming coccidian protozoan parasites with a wide host range, including rodents. In this study, *Sarcocystis* spp. samples were isolated from *Bandicota indica*, *Rattus argentiventer*, *R. tiomanicus* and *R. norvegicus* across five provinces of Thailand. Two major groups of *Sarcocystis* cysts were determined in this study: large and small cysts. By sequence comparisons and phylogenetic analyses based on the partial sequences of 28S ribosomal DNA, the large cysts showed the highest identity value (99%) with the *S. zamani* in GenBank database. While the small cysts could be divided into 2 groups of *Sarcocystis*: *S. singaporensis* and presupposed *S. zuoi*. The further analysis on 18S rDNA supported that the 2 isolates (S2 and B6 no.2) were as identified as *S. singaporensis* shared a high sequence identity with the *S. singaporensis* in GenBank database and the unidentified *Sarcocystis* (4 isolates, i.e., B6 no.10, B6 no.12, B10 no.4 and B10 no.7) showed 96.3–99.5% identity to *S. zuoi* as well as high distinct identity from others *Sarcocystis* spp. (≤93%). The result indicated that these four samples should be *S. zuoi*. In this study, we provided complete sequence of internal transcribed spacer 1 (ITS1), 5.8S rDNA and internal transcribed spacer 2 (ITS2) of these three *Sarcocystis* species and our new primer set could be useful to study the evolution of *Sarcocystis*.

KEY WORDS: phylogenetic analysis, rodent intermediate hosts, *Sarcocystis* spp.

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Sarcocystis species are cyst-forming coccidia (Alveolata: Apicomplexa: Coccidia) and have two host life cycles between the sexual and asexual stages (heteroxenous life cycles), using the predator–prey relationship [1, 7]. During the sexual stage, sporocysts multiply in the small intestine of the definitive host (predator) and are excreted in the definitive host's feces, and the asexual stage (sarcocysts) develops in the muscles of the intermediate host [15]. *S. zamani* and *S. singaporensis* use rodents of the genera *Bandicota* and *Rattus* as intermediate hosts and snakes (*Python reticulatus*) as definitive hosts [11, 17]. During the life cycle of *S. zuoi*, Norway rats (*R. norvegicus*) are used as intermediate hosts and snakes (*Elaphe*) as definitive hosts [1, 9]. Several species in this group, such as *S. neurona* and *S. canis*, can cause systemic illness in many species of animals [6], and *S. nesbitti* is a causative agent of acute human muscular sarcocystosis in Malaysia [1, 2, 10].

Basically, the identification of this genus has been based on the cyst morphology and the molecular method or a combination of these methods [11, 15]. Phylogenetic analyses on the members of this group have largely been based on nuclear ribosomal DNA (rDNA), such as the 18S rDNA and 28S rDNA nucleotide sequences, to reconstruct the evolutionary relationship between the organisms [13, 16, 17]. The 18S rDNA sequences are becoming possible to improve and refine the current taxonomic system which is based mainly on phenotypic characters [5], while the 28S rDNA helped to resolve some relationships and confirm other postulated from the 18S rDNA analyses [13]. The internal transcribed spacer (ITS1 and ITS2) regions can be useful for the identification and differentiation of closely related *Sarcocystis* species, such as *S. calchasi* and *S. columbae*, which are found in wood pigeons (*Columba palumbus*) [14, 16]. However, there are still a few complete sequences of ITS1, 5.8S rDNA and ITS2 data in *Sarcocystis* spp.

*Correspondence to: Sukmak, M.: fvetmksu@gmail.com

Table 1. The *Sarcocystis* spp. isolates used in this study

Species	Accession no.	Voucher no.	Country	Sampling location	Locality coordinating	Size of the cyst	Intermediate host
S. zamani	KU244520	B23 no.3	Thailand	TaaSae, Chumphon	47P 0543833 1207973	Large cyst	*Rattus tiomanicus*
	KU244521	BJ no.1	Thailand	TaaSae, Chumphon	47P 054090 1212120	Large cyst	*Rattus tiomanicus*
	KU244522	DOA02	Thailand	Krasaesin, Songkhla	47N 649220 844496	Large cyst	*Rattus argentiventer*
	KU244523	DOA05	Thailand	Krasaesin, Songkhla	47N 649220 844496	Large cyst	*Rattus argentiventer*
	KU244524	no.1	Thailand	Hua Hin, PraChuap Khiri Khan	47P 604044 1389620	Large cyst	*Bandicota indica*
	KU244525	no.2	Thailand	Hua Hin, PraChuap Khiri Khan	47P 604044 1389620	Large cyst	*Bandicota indica*
	KU244526	no.15	Thailand	BanMi, Lopburi	47P 659640 1669000	Large cyst	*Rattus argentiventer*
	KU244527	no. 13.2[a)]	Thailand	Bangkhen, Bangkok	47P 672509 1534355	Large cyst	*Rattus norvegicus*
	KU244528	R.t	Thailand	TaaSae, Chumphon	47P 0543090 1212120	Large cyst	*Rattus tiomanicus*
S. zuoi	KU341120	B10 no.4	Thailand	TaaSae, Chumphon	47P 0542724 1210951	Small cyst	*Rattus tiomanicus*
	KU341119	B6 no.12	Thailand	TaaSae, Chumphon	47P 0543038 1210361	Small cyst	*Rattus tiomanicus*
	KU341121	B10 no.7	Thailand	TaaSae, Chumphon	47P 0542724 1210951	Small cyst	*Rattus tiomanicus*
	KU341118	B6 no.10	Thailand	TaaSae, Chumphon	47P 0543038 1210361	Small cyst	*Rattus tiomanicus*
S. singaporensis	KU341122	S2	Thailand	Hua Hin, PraChuap Khiri Khan	47P 604044 1389620	Small cyst	*Bandicota indica*
	KU341123	B6 no.2	Thailand	TaaSae, Chumphon	47P 0543038 1210361	Small cyst	*Rattus tiomanicus*

a) Samples maintained in our laboratory, Entomology & Zoology Research Group, Plant Protection Research & Development Office, Department of Agriculture, Chatuchuk, Bangkok, Thailand.

Table 2. PCR forward (F) and reverse (R) primers used to amplify various DNA regions in this study

Regions	Primer name	Sequences 5′ to 3′	T (°C)[a)]	Reference
18S rDNA	18S-Fw	AGC CAT GCA TGT CTA AGT ATA AG (23 bp)	60	Modified from [20]
	1471-Rw	TAT CCC CAT CAC GAT GCA TAC (21 bp)	60	Modified from [20]
	18S-3 (1251) w	GTT AAC GAA CGA GAC CTT AAC C (22 bp)	60	This study
	zam-1717w	GTG AAC CTT AAC ACC TAG AGG AAG GA (26 bp)	76	This study (for *S. zamani*)
ITS1-5.8S rDNA-ITS2	sar 5.8-1w	CAA TGG ATG TCT TGG CTC GCG CAA (24 bp)	68	This study
	sar 5.8-2w	C AAA GAC ACT GAA ACA GAC GTG CTG (25 bp)	68	This study
	zam-1870w	TCT CCG TTG CCG CCA GGA ACG TGA GAC (27 bp)	78	This study (for *S. zamani*)
28S rDNA	28S-1 (001) w	TAC CCG CTG AAC TTA AGC ATA TTA T (25 bp)	64	This study
	28S-4 (290) w	TTT GCG CTC AAA TCC CGA TGA A (22 bp)	60	This study
	28S-2 (1880) w	CTA GAG ACT GTT CCA CGC AGG CTT A (25 bp)	64	This study

a) T refers to annealing temperature,Yang *et al*. [20] .

MATERIALS AND METHODS

In 2013 and 2014, a total of 15 wild rodents (Rodentia: Muridae) of the genera *Bandicota* and *Rattus* were used in this study. Species identification of rodents was performed by rodents' specialist (Hamarit; personal communication). All rodents examined were obtained as fresh carcasses from several farmers in five provinces of Thailand (Table 1), because the rodents were considered as pest animals in farms. Sarcocysts were isolated from the abdominal muscles or all striated muscles and investigated under a light microscope (LM) (Olympus BX40, Tokyo, Japan) at the total magnification of 50×, 100× and 400×, respectively. The observed sarcocysts were individually dissected, placed in labeled 1.5 m*l* tubes and then kept frozen at −20°C until the DNA extraction process. Total genomic DNA (gDNA) was extracted using the QIAmp DNA mini kit (Qiagen, Hilden, Germany) according to the manufacturer's tissue protocol recommendations. For the polymerase chain reaction (PCR), we designed primers based on 28S rDNA nucleotide sequences of *S. zamani* and *S. singaporensis* (AF237616 and AF237617) from previous studies [13], and the primers of 18S rDNA, ITS1-5.8S rDNA-ITS2 and 28S rDNA (Table 2 and Fig. 1) were employed for the amplification of *Sarcocystis* species in this study. Each PCR reaction mixture contained 3 μl of the gDNA template solution, 10× HF buffer, 200 μM of dNTPs, 10 μM of each primers and 1 U Phusion hotstart *Taq* DNA polymerase (Thermo Scientific, Waltham, MA, U.S.A.). The PCR was performed in a Peltier thermal cycler machine (Bio-Rad, Hercules, CA, U.S.A.), model PTC-200, the program of which was 98°C for 30 sec, followed by 40 cycles of 98°C for 30 sec, 60°C for 30 sec and 72°C for 90 sec, ending with a final extension of 72°C for 5 min. The PCR products were purified using the Gel Elution Kit (GeneMark, Taipei, Taiwan), according to the manufacturer's protocol recommendations. The PCR products were sent to First BASE laboratories, Shah Alam, Malaysia for sequencing. The obtained sequences were aligned by Muscle in the MEGA 6 software [19] and edited to build a consensus sequence for each segment, using the BioEdit version 7.0 [8]. The nucleotide sequences of the *Sarcocystis* species (15 isolates) in this study were deposited in the GenBank database with the following accession numbers: *S. zamani*: KU244520–KU244528, *S. singaporensis*: KU341122–KU341123 and *S. zuoi*: KU341118–KU341121. Phylogenetic relationship was calculated

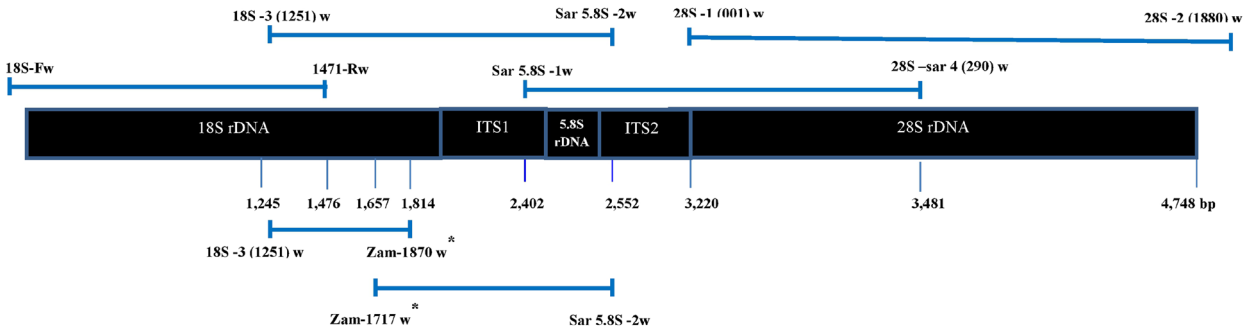

Fig. 1. Primers map of the 18S rDNA partial sequence, ITS1-5.8S-ITS2 complete sequence, 28S rDNA partial sequence. The map has roughly been draw to scale. The primers positions were indicated based on KU244520 *Sarcocystis zamani* (isolated B23 no.3) length 4,748 bp. *Primer zam-1717w and zam-1870w were specifically used for nucleotides sequencing of *S. zamani*.

by using three different methods, including neighbor-joining (NJ), maximum parsimony (MP) and maximum likelihood (ML). The phylogenetic trees were constructed and selected the best model using Find Best DNA Model test performed via MEGA 6 software [19]. The Hasegawa-Kishino-Yano model was used for ITS1-5.8S rDNA-ITS2 full length region. For 18S rDNA and 28S rDNA, partial sequences were performed using the Kimura 2-parameter distance model and Tamura 3-parameter, respectively. The three phylogenetic trees were tested using 1,000 bootstrap replicates, and the sequences of *Toxoplasma gondii* (X75429) and *Besnoitia besnoiti* (DQ227419) were used as the out group.

RESULTS

By LM, two major groups of *Sarcocystis* cysts were determined in this study: large and small cysts (Table 1). The morphology of large cysts (N=9) looks like grains of rice with thin and delicate wall, and they could be seen by the naked eye. Microscopic examination proved those of large cysts that were similar to *S. zamani* [3]. The small cysts showed two types of sarcocysts; the first type (N=2) had thick wall cysts and fine surface villi. These microscope findings suggest that the characteristics of the tissue cysts were similar to those of *S. singaporensis*, in accordance with the previous studies [3]. The second type of small cysts (N=4) had thin wall cyst isolated from four isolations of *R. tiomanicus*. Microscopically, they were determined as an unidentified species of *Sarcocystis* spp., and the cyst surface was distinguished from that of *S. singaporensis*.

Both of the small cysts were smaller than those of *S. zamani* and difficult to be seen with the naked eye. In this study, the phylogenetic tree construction based on partial 18S rDNA, 28S rDNA and full-length ITS1- 5.8S rDNA-ITS2, sequences of sarcocysts isolated from 14 wild rodents and 1 laboratory rat in Thailand [N=15] can be separated into three groups (groups A–C) under NJ, ML and MP criteria (Figs. 2–4). These three *Sarcocystis* species from fifteen isolates in this study (*S. zamani*, *S. singaporensis* and *S. zuoi*) used rodent and snake as intermediate and definitive hosts, respectively. The PCR product length of 18S rDNA and 28S rDNA was approximately 1.4 and 1.5 kb, respectively. The PCR product of ITS1-5.8S rDNA-ITS2 region was found three sizes in *S. zamani* (approximately 560, 890 and 1,000 bp) and two sizes in *S. zuoi* and *S. singaporensis* (estimated 1.0 and 1.3 kb, respectively). The phylogenetic tree inferred with the partial sequences of 28S rDNA was reconstructed using the NJ method supported an identical tree to that of the ML and MP analyses. Groups A to C were placed in the reptile/mammal life cycle (used snake/rodent as definitive and intermediate hosts, respectively) and were within these three groups (A–C) with high bootstrap values identified and categorized based on the differences on morphology of cysts: *S. zamani* group (group A: large cysts), *S. zuoi* group (group B: small thin wall cysts) and *S. singaporensis* group (group C: small thick wall cysts and surface villi) (Fig. 2). The tree topology of *S. zamani* group from our samples (N=9) was placed in the same cluster as group A and could also be divided into two sub-clusters, i.e., i) seven samples (KU244520-KU244523 and KU244526-KU244528) which were isolated from three different *Rattus* spp. and ii) two samples (KU244524-KU244525) which were isolated from *B. indica*, that shared the highest identity value (99%) with the *S. zamani* sequence (GenBank AF237616). The six small cyst samples were classified into two groups (B and C). The two small cysts, isolates S2 (KU341122) and B6 no.2 (KU341123), were placed in group C with the *S. singaporensis* sequence in the GenBank database (AF237617). While the four isolates, B10 no.4 (KU341120), B6 no.12 (KU341119), B6 no.10 (KU341118) and B10 no.7 (KU341121), from *R. tiomanicus* in this study were placed in group B. When the four isolates from *R. tiomanicus* (group B) were compared with *S. zamani* (group A) and *S. singaporensis* (group C), the sequences identities were found to be 90.3–91.8% and 87.2–91.7%, respectively. Thus, the results show that the four *Sarcocystis* isolates from *R. tiomanicus* have significant genetic differences in the 28S rDNA sequence and can be separated from the *S. zamani* and *S. singaporensis* species. A total of 15 isolates in this study were phylogenetically fallen into three clades (Fig. 2).

In the phylogenetic analysis based on the partial 18S rDNA sequences (Fig. 3), all isolates of *S. zamani* in this study (KU244520-KU244528) were placed in the group A as well as unidentified *Sarcocystis* species isolated from the reticulated python *(Braghammerus reticulatus*) (KC878485) with 97.8–98.6% identity. Group B is divided into two subgroups (BI and BII). Subgroup BI can be separated into two clusters based on the bootstrap values, consisted of the four isolates (KU341118–KU341121) from *R.*

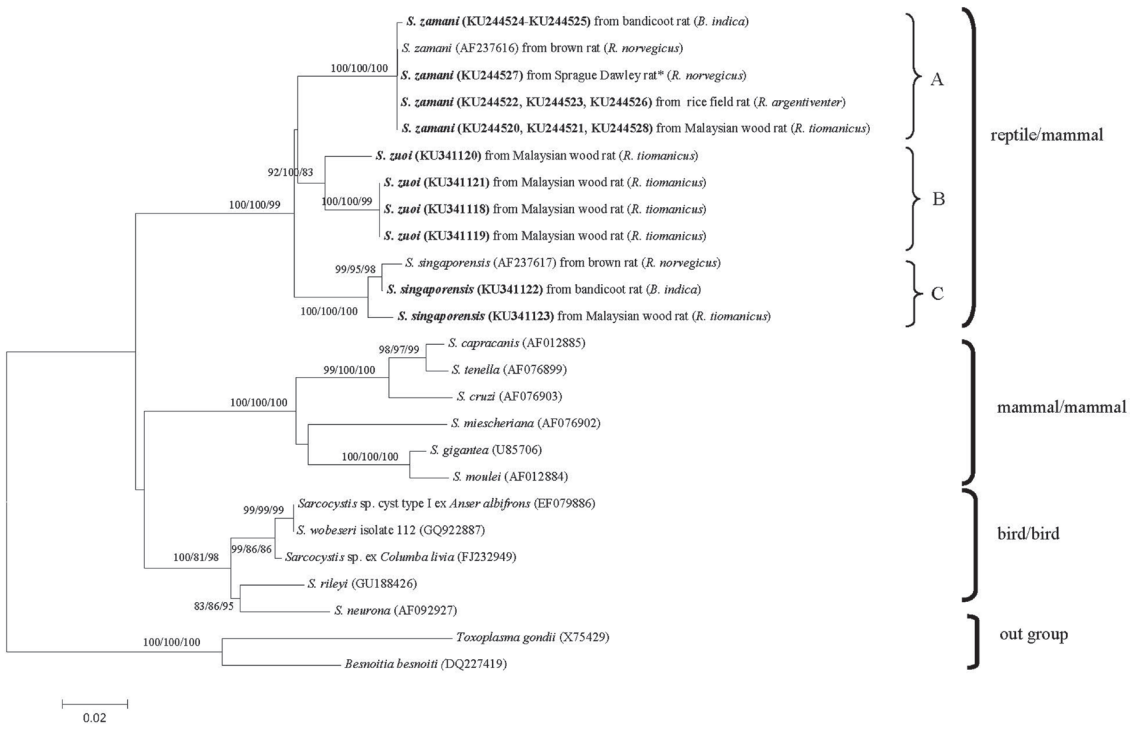

Fig. 2. Phylogenetic tree of *Sarcocystis* spp. based on 28S rDNA partial sequences (1 kb) with related sequences obtained from GenBank database, reconstructed using NJ/ML/MP method. The group (A–C) of *Sarcocystis* species is identified as reptile/mammal life cycles. Bootstrap support (1,000 replicates) shows at each node following three different methods, respectively, and the bootstrap values below 70% are not shown. Scale bar is according to the branch length and rooted on *Toxoplasma gondii* (X75429) and *Besnoitia besnoiti* (DQ227419). *This isolation obtained from Sprague Dawley rat that was routinely used in laboratory of entomology & Zoology Research Group, Plant Protection Research & Development Office, Department of Agriculture, Thailand (personal communication).

tiomanicus and *S. zuoi* sequences in the GenBank (JQ029112 and JQ029113) from the king rat snake (*Elaphe carinata*) in China [9], LC054267 from the beauty snake (*Elaphe taeniura*) in Japan [1], and KC878487 and KC878488 from the Malayan keeled rat snake (*Ptyas carinata*) in Malaysia. Subgroup BII was identified as *S. clethrionomyelaphis* which comprised of *Sarcocystis* sp. (KC201639) from the green tree python (*Morelia viridis*) and *S. clethrionomyelaphis* (KP057504) from the beauty snake (*E. taeniura*). The four isolates from *R. tiomanicus* in this study (KU341118–KU341121) were located in the same group as *S. zuoi*, JQ029112 and JQ029113 and closely related with other *S. zuoi* sequences in the GenBank database. Sequences identity value of the four isolates (KU341118–KU341121) in this study and *S. zuoi* sequences in GenBank database accounted for 96.3–99.5%, also showed a distinct identity from others *Sarcocystis* spp. (93% or less). Group C (the *S. singaporensis* species) was clustered into two minor groups (CI–CII). For the isolate S2 (KU341122) from the bandicoot rat, *B. indica*, and B6 no.2 (KU341123) from the Malaysian wood rat, *R. tiomanicus*, in the present study, the phylogenetic placement of these two isolates as inferred from figures as 18S rDNA (Fig. 3) and 28S rDNA (Fig. 4) sequences was placed in the group of *S. singaporensis* species from the GenBank database (group C). Sequences identity of *S. singaporensis* in this study (KU341122-KU341123) had 99.8 and 100% identity (data not shown) to the closet sequences of *S. singaporensis* in the GenBank, AF434056 and AF434058, respectively for 18S rDNA. While 28S rDNA sequence data of both isolates, KU341122-KU341123, were 98.4 and 96.3% identical with *S. singaporensis* from GenBank (AF237617) and were higher than compared with *S. zamani* (88.3–94.5 and 90.3–91.8%) and *S. zuoi* (87.6–93.6% and 87.2–91.7%). The results of sequence homology indicated that the two isolates, KU341122 and KU341123, were identified as *S. singaporensis*. The two fragments of 18S rDNA sequences in the present study (KU341122 and KU341123), were placed in CI subgroup and closely related with the CII subgroup, where the five sequences in CII used the Malayan brown pit viper, *(Ovophis convictus*) as definitive hosts [1]. Although our phylogenetic tree can divide our sequences into different subgroups (BI/BII and CI/CII), but the certain taxon was not supported by high bootstrap value, indicating ambiguity at subgroup level for each tree. Other *Sarcocystis* species isolated from snake definitive host was comprised of *S. nesbitti* and *S. atheridis* which be clustered with the mammal/mammal life cycle (e.g. *S. tenella*, *S. capracanis*, *S. cruzi*, *S. gigantea*, *S. moulei*, *S. buffalonis* and *S. hirsuta*). Three *Sarcocystis* species, including *S. dispersa*, *S. albifronsi* and *Sarcocystis* sp. ex *Columba livia*, were placed in the cluster bird/bird life cycle and can be divided into two subgroups based on the high bootstrap values (Fig. 3).

The trees obtained from the complete ITS1-5.8S rDNA-ITS2, partial 18S rDNA and 28S rDNA nucleotide sequences of *S. zamani*, *S. zuoi* and *S. singaporensis* in this study were estimated at 4 kb in length, The different constructed methods (NJ, ML and MP) mostly supported the same branching pattern for each region. However, the differences of branching pattern can be observed

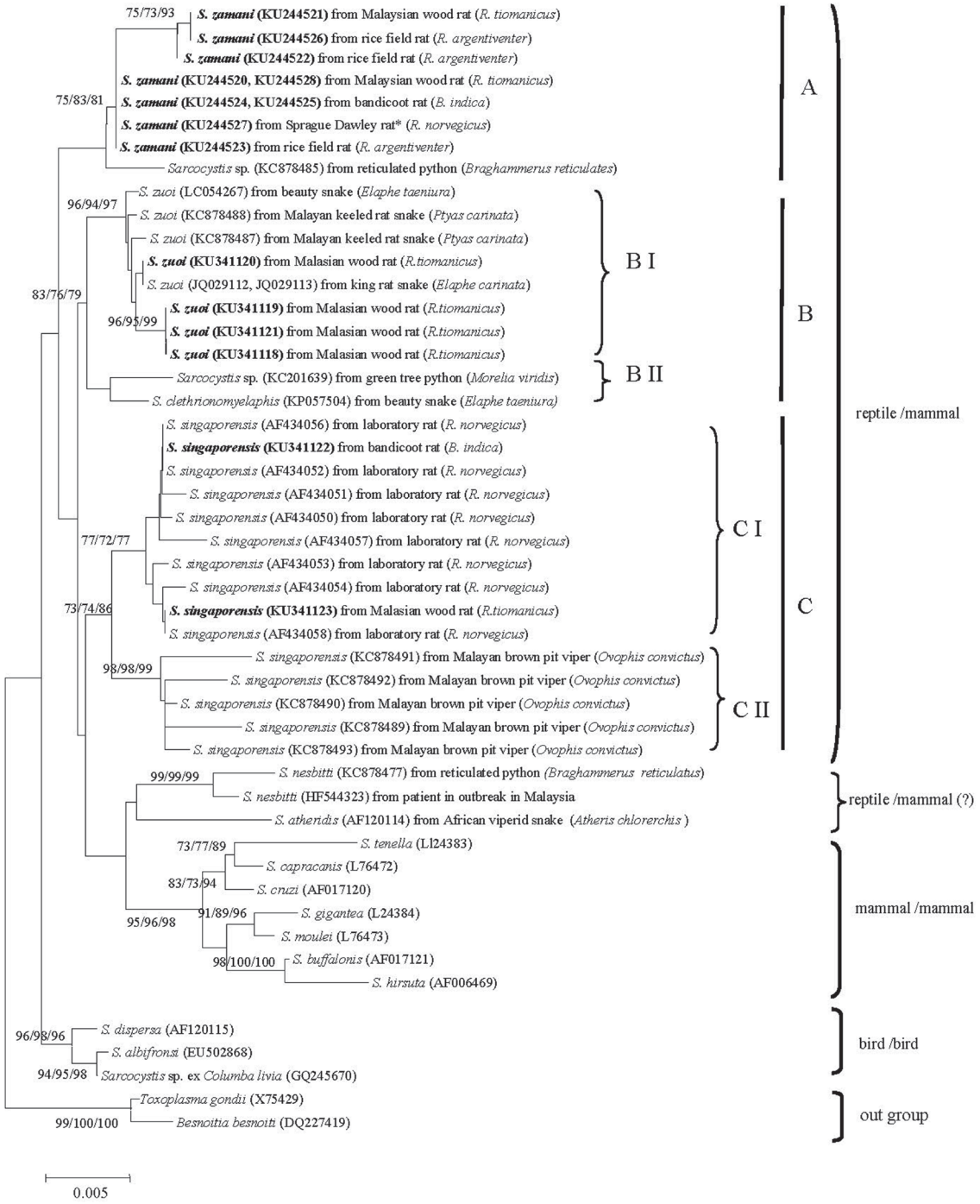

Fig. 3. Phylogenetic tree of *Sarcocystis* spp. based on 18S rDNA partial sequences (800 bp) and related sequences obtained from GenBank database, reconstructed using NJ/ML/MP method. The group (A–C) of *Sarcocystis* species is identified as reptile/mammal life cycles. Subgroup (BI, BII and CI, CII) was classified by their diversity within species level. Bootstrap support (1,000 replicates) shows at each node following three different methods, respectively, and the bootstrap values below 70% are not shown. Scale bar is according to the branch length and rooted on *Toxoplasma gondii* (X75429) and *Besnoitia besnoiti* (DQ227419). *This isolation obtained from Sprague Dawley rat routinely used in laboratory of Entomology & Zoology Research Group, Plant Protection Research & Development Office, Department of Agriculture, Thailand (personal communication). (?) These species were previously reported as reptile/mammal life cycles, but are genetically placed in mammal/mammal life cycles cluster.

between phylogenetic trees from different gene. The sequence homology for intraspecies level in our studied was 98.6–99 , 83.2–98.1 and 95% for *S. zamani, S. zuoi* and *S. singaporensis*, respectively (data not shown). Conversely, for interspecies level, the sequence homology among three species was ≤80% identity (data not shown). The topology of the phylogenetic tree divided the

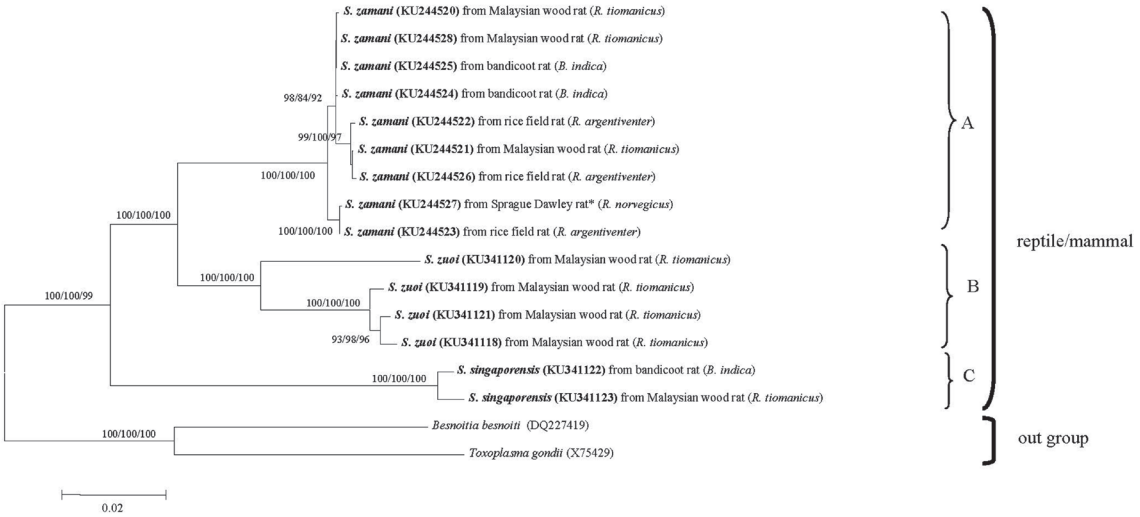

Fig. 4. Phylogenetic tree of *Sarcocystis* spp. based on 18S rDNA partial sequences, internal transcribed spacer 1 (ITS1) −5.8S rDNA −internal transcribed spacer 2 (ITS2) complete sequences and 28S rDNA partial sequences (3.7 kb) with related sequences from GenBank database and reconstructed using NJ/ML/MP method. The group (A–C) of *Sarcocystis* species is identified as reptile/mammal life cycles. Bootstrap support (1,000 replicates) shows at each node following three different methods, respectively, and the bootstrap values below 70% are not shown. Scale bar is according to the branch length and rooted on *Toxoplasma gondii* (X75429) and *Besnoitia besnoiti* (DQ227419). *This isolation obtained from Sprague Dawley rat routinely used in laboratory of Entomology & Zoology Research Group, Plant Protection Research & Development Office, Department of Agriculture, Thailand (personal communication).

15 isolates of *Sarcocystis* species in this study into five clusters, seven isolates of *S. zamani* (KU244520, KU244521-KU244522, KU244524-KU244526 and KU244528), two isolates of *S. zamani* (KU244523, KU244527), one isolate of *S. zuoi* (KU341120), three isolates of *S. zuoi* (KU341118-KU341119 and KU341121) and two isolates of *S. singaporensis* (KU341122-KU341123), respectively (Fig. 4).

DISCUSSION

The identification and classification of *Sarcocystis* species have been based on morphology and biological data [5, 11, 16, 18], such as details of cyst wall, villi, host specificity and their molecular characteristics. Morphological characteristic of the cyst wall and the life cycle are two key criteria of conventional classification for *Sarcocystis*. In addition, the study of speciation by using molecular markers, such as 18S rDNA and ITS1 regions, is more efficient and accurate to classify the *Sarcocystis* species, and it can resolve some questions and differentiate the closely related species of the Sarcocystidae.

The present study based on partial sequence of the 28S rDNA sequences revealed that *S. zamani* has a close relationship to the *S. singaporensis* and *S. zuoi* groups, place in "reptile/mammal life cycle" cluster and separate from the mammal/mammal and the bird/bird life cycle cluster (Fig. 2) as well as described in a previous study [1, 11]. Since these 3 species used snakes as definitive hosts, thus our finding indicated that the *Sarcocystis* species in this group are snake-transmitted species.

Our phylogenetic tree based on partial sequence of the 18S rDNA sequence revealed the four isolates (KU341118–KU341121) from *R. tiomanicus* are closely related to *S. zuoi* (JQ029112 and JQ029113) isolated from king rat snake (*E. carinata*) (Fig. 3). They were placed in *S. zuoi* group with high identity value (96.3–99.5%) and demonstrated clearly different from *S. zamani* and *S. singaporensis*, indicating that these four isolates may be *S. zuoi* species. So far, the 18S rDNA sequence data have favored the co-evolution of *Sarcocystis* species in snakes, with a definitive host rather than intermediate host [5]. Therefore, our study hypothesized that the *Elaphe* or *Ptyas* snake may be the definitive host of the four isolates from *R. tiomanicus* in this study. For *S. singaporensis* (group C), our analysis found high genetic divergent among this species and can divide into two subgroups (CI-CII; Fig. 3). *S. singaporensis* has been considered as highly pathogenic caused lethal death in rat and widely dispersed across Southeast Asia. The intermediate host included *Rattus* and *Bandicota* species as well as this study. Many studies reported that python was the natural definitive host [11, 17]. In addition, the viper (*O. convictus*) was considered as a definitive host of *S. singaporensis* according to Abe, 2015 [1] and clustered in the "CII" subgroup (Fig. 3). Although, the bootstrap value (73/74/86) provided low clustering supported divergence between "CI" and "CII", however, accordance with previous studies [11, 17], can be implied that *S. singaporensis* within the "CI" subgroup and two isolates in this study (KU341122 and KU341123), used a python as a definitive host.

The phylogenetic analyses based on the partial 18S rDNA sequences (Fig. 3) demonstrated that *S. nesbitti* (KC878477) and *S. atheridis* (AF120114) were placed in the same cluster of *Sarcocystis* species that have mammal/mammal life cycle. However, *S. nesbitti* and *S. atheridis* were considered to use snake as a definitive host [1, 11], indicating that the phylogenetic position of the *Sarcocystis* species did not correlate with their definitive hosts/life cycle, according to the previous studies [18].

The phylogenetic tree construction using 18S rDNA, 28S rDNA partial sequences and ITS1- 5.8S rDNA-ITS2 full-length sequences region, can be divided into 4 subgroups, among the 13 isolates of *S. zamani* and *S. zuoi* (Fig. 4). To date, the ITS1-5.8S rDNA-ITS2 region was considered to evolve much faster than other rDNA regions [4, 12]. Thus, the phylogenetic analyses based on ITS1-5.8S rDNA-ITS2 sequences would be useful for distinguishing among the same species or sub-species level better than the more slowly evolving, such as 18S rDNA and 28S rDNA region, and are better to use among different species level. In conclusion, this study is the first report on the molecular characteristics of the full-length ITS1-5.8S rDNA-ITS2, partial 18S and 28S rDNA nucleotide sequences of *S. zamani*, *S. zuoi* and *S. singaporensis* in the GenBank database and to reveal the genetic diversity of the three species distributed in Thailand. However, the definitive host (snakes) was not verified for *S. zuoi* species in this study. In further studies, more feces samples of wild snakes containing sporocysts should be collected, and the transmission experiment must be conducted to clarify the definitive host of *S. zuoi* and other *Sarcocystis* species in Thailand. Therefore, not only the analysis sequences in one region gene, but also the comparison in other regions are insufficient for the classification of *Sarcocystis* species. Nevertheless, further sampling from a broader geographic area in Thailand and combined data will clarify the question of genetic variation and genetic diversity in this species. The benefits derived from this study can be helpful for further research of *Sarcocystis* species in snake definitive hosts and application for classification to prevent protozoa infection in both human and animals.

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