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▣ ORIGINAL ARTICLE

Morphology and Molecular Phylogeny of *Raillietina* spp. (Cestoda: Cyclophyllidea: Davaineidae) from Domestic Chickens in Thailand

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Abstract: *Raillietina* species are prevalent in domestic chickens (*Gallus gallus domesticus*) in Phayao province, northern Thailand. Their infection may cause disease and death, which affects the public health and economic situation in chicken farms. The identification of *Raillietina* has been based on morphology and molecular analysis. In this study, morphological observations using light (LM) and scanning electron microscopies (SEM) coupled with molecular analysis of the internal transcribed spacer 2 (ITS2) region and the nicotinamide adenine dinucleotide dehydrogenase subunit 1 (ND1) gene were employed for precise identification and phylogenetic relationship studies of *Raillietina* spp. Four *Raillietina* species, including *R. echinobothrida, R. tetragona, R. cesticillus*, and *Raillietina* sp., were recovered in domestic chickens from 4 districts in Phayao province, Thailand. LM and SEM observations revealed differences in the morphology of the scolex, position of the genital pore, number of eggs per egg capsule, and rostellar opening surface structures in all 4 species. Phylogenetic relationships were found among the phylogenetic trees obtained by the maximum likelihood and distance-based neighbor-joining methods. ITS2 and ND1 sequence data recorded from *Raillietina* sp. appeared to be monophyletic. The query sequences of *R. echinobothrida, R. tetragona, R. cesticillus*, and *Raillietina* sp. were separated according to the different morphological characters. This study confirmed that morphological studies combined with molecular analyses can differentiate related species within the genus *Raillietina* in Thailand.

Key words: *Raillietina echinobothrida, Raillietina tetragona, Raillietina cesticillus, Raillietina* sp., morphology, phylogeny, ITS2, ND1, chicken, Thailand

INTRODUCTION

Among the davaineid genera, the most common is *Raillietina* Fuhrmann, 1920 with about 295 species reported from avian and mammalian hosts, including humans [1]. Their infections are highly pathogenic in domestic chickens (*Gallus gallus domesticus*), for example, in Phayao province [2]. *Raillietina* species are found in the jejunum and ileum of the definitive host and can cause reduced growth, emaciation, weakness, and digestive tract obstruction [3], whereas their larval stage (cyticercoid) resides in various invertebrate intermediate host, such as ants, beetles, small mini-wasps, or termites [4,5]. The genus *Raillietina* is divided into 4 subgenera; *Raillietina* Fuhrmann, 1920, *Paroniella* Fuhrmann, 1920, *Skrjabinia* Fuhrmann, 1920, and *Fuhrmann* Fuhrmann, 1920 [1].

The species identification of *Raillietina* has been based on the morphology and molecular study results. The morphological characters used for distinguishing *Raillietina* spp. include differences in the size and shape of the scolex, morphology of the rostellum (armed with either a single row or double rows of hooks) and suckers (armed or unarmed), the position (unilateral or irregularly alternating) and number of genital pores per segment, and the number of the eggs within an egg capsule in gravid proglottids [1,6,7]. However, the morphological characters of *Raillietina* spp. show marked variations within and between species. Therefore, it is difficult to identify them only based on the morphology. Instead, molecular approaches, in combination with morphological analyses, became the most effective and accurate method for identification of helminth parasites. With regard to the molecular techniques, conventional PCR and HAT-RAPD have been used to differentiate among *Raillietina* group obtained from domestic chickens in

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Thailand [2]. However, there are many DNA regions useful for identification of helminths and discrimination between species with similar morphologies. Especially, internal transcribed spacer 2 (ITS2) in nuclear gene and nicotinamide adenine dinucleotide dehydrogenase subunit 1 (ND1) in mitochondrial gene are commonly used because of their high variation rates. In addition, the gene sequences of these locations have been developed for molecular phylogenetic studies [8-11].

In the present study, we examined the ITS2 and ND1 regions to infer the phylogenetic relationships of *Raillietina* spp. from domestic chickens (*Gallus gallus domesticus*) in Phayao province, Thailand, in combination with morphological analyses using light (LM) and scanning electron microscopes (SEM).

MATERIALS AND METHODS

Sample collection

Live tapeworms (*Raillietina* spp.) were obtained from the small intestine of naturally infected domestic chickens from 4 districts (Chiang Kham, Chun, Dok Khamtai, and Mae Chai) in Phayao province, Thailand. The specimens were flattened and fixed in 4% formalin for light microscopic (LM) studies and prefixed in 2.5% glutaraldehyde for scanning electron microscopic (SEM) studies. For molecular analysis, the specimens were rinsed several times with tape water and then frozen at -20℃ immediately for later DNA extraction.

Morphological observations

For LM observations, the specimens were prepared for permanent slides. All permanent slides of each species were drawn as figures by using a drawing tube. For SEM observations, the specimens were post-fixed with 1% osmium tetroxide. They were dehydrated in a graded series of ethanol (10%, 20%, 30%, 50%, 70%, 85%, 95%, and absolute), then transferred into acetone, and finally dried in a critical-point dryer. The specimens were mounted on stubs, gold-coated, and observed with JEOL-JSM5400LV scanning electron microscope (Tokyo, Japan) at an accelerating voltage of 10-15 kV.

DNA extraction

Genomic DNA was extracted by using Chelex solution. In brief, 150 µl of 5% Chelex (Fluka) solution containing 10 µl of proteinase K (Sigma-Aldrich, St. Louis, Missouri, USA) at a concentration of 20 mg/ml was added to approximately 20 mg of the cestode tissue. It was then heated to 55℃ for 1 hr, followed by gentle vortexing and heating at 95℃ for 30 min, again followed by gentle vortexing. The mixture was centrifuged at 13,000 g for 1 min. The supernatant was collected and stored at - 20℃ until used.

Amplification of ITS2 region

The ITS2 region was amplified by using the primers, forward 3S (5′-GGT ACC GGT GGA TCA CTC GGC TCG TG-3′) and reverse BD2 (5′-TAT GCT TAA ATT CAG CGG GT-3′). The PCR conditions were as follows: 2 min initial denaturation at 94℃, followed by 35 cycles of 1 min DNA denaturation at 94℃, 1 min primer annealing at 57℃, 1 min extension at 72℃, and a final extension at 72℃ for 7 min.

The amplification product was checked using agarose gel electrophoresis with ethidium bromide staining. All PCR products were purified using Cleanup PCR Kit (Sigma-Aldrich) and were subjected to sequencing.

Amplification of ND1 gene

The ND1 gene was amplified by using the primers, JB11 (5′-AGA TTC GTA AGG GGC CTA ATA -3′) as the forward primer and JB12 (5′-ACC ACT AAC TAA TTC ACT TTC -3′) as the reverse primer. The PCR conditions were as follows: 2 min initial denaturation at 94℃, followed by 39 cycles of 30 sec DNA denaturation at 94℃, 20 sec primer annealing at 48℃, 1 min extension at 72℃, and a final extension at 72℃ for 10 min.

The amplification product was checked using agarose gel electrophoresis with ethidium bromide staining to visualize the ND1 products. All PCR products were purified using Cleanup PCR Kit (Sigma-Aldrich) and were subjected to sequencing.

Phylogenetic analysis

Our data sequence, ITS2 product revealed the size of 944, 1,008, 813 and 588 bp with flanking region of 5.8S and 28S in *R. echinobothrida*, *R. tetragona*, *R.cesticillus* and *Raillietina* sp., respectively. For the ND1 product revealed the size of partial sequence of 489 bp in all four species of *Raillietina*. The data in this study included the sequences from the representative group of *Raillietina* in GenBank (Tables 1, 2), and then were aligned using ClustalW. The phylogenetic tree was constructed for each gene using MEGA version 6.0 with neighbor-joining (NJ) and maximum-likelihood (ML) methods. The statistics supported for branches were tested using 1,000 bootstrap replicates.

Table 1. Helminth species used in this study and GenBank accession numbers for their corresponding ITS2 sequence

Species	Host	Country	GenBank accession no.	Family
Raillietina echinobothrida[a]	*Gallus gallus domesticus*	Thailand	-	Davaineidae
Raillietina tetragona[a]	*Gallus gallus domesticus*	Thailand	-	Davaineidae
Raillietina cesticillus[a]	*Gallus gallus domesticus*	Thailand	-	Davaineidae
Raillietina sp.[a]	*Gallus gallus domesticus*	Thailand	-	Davaineidae
Raillietina australis	*Dromaius novaehollandiae*	Australia	AY382317	Davaineidae
Raillietina chiltoni	*Dromaius novaehollandiae*	Australia	AY382319	Davaineidae
Raillietina beveridgei	*Dromaius novaehollandiae*	Australia	AY382318	Davaineidae
Raillietina dromaius	*Dromaius novaehollandiae*	Australia	AY382320	Davaineidae
Taenia saginata	Cattle	China	AY825542	Taeniidae
Taenia pisiformis	Rabbit	China	JX317675	Taeniidae
Taenia hydatigena	Dog	China	FJ886761	Taeniidae
Hymenolepis diminuta	Mouse	Japan	AB494474	Hymenolepididae
Hymenolepis nana	Mouse	Mexico	HM536189	Hymenolepididae
Hymenolepis microstoma	Hmic	Japan	AB494478	Hymenolepididae
Diphyllobothrium nihonkaiense[b]	*Homo sapiens*	Japan	AB288369	Diphyllobothriidae

Table 2. Helminth species used in this study and GenBank accession numbers for their corresponding ND1 sequence

Species	Host	Country	GenBank accession no.	Family
Raillietina echinobothrida[a]	*Gallus gallus domesticus*	Thailand	-	Davaineidae
Raillietina tetragona[a]	*Gallus gallus domesticus*	Thailand	-	Davaineidae
Raillietina cesticillus[a]	*Gallus gallus domesticus*	Thailand	-	Davaineidae
Raillietina sp.[a]	*Gallus gallus domesticus*	Thailand	-	Davaineidae
Raillietina sonini	*Dendrocopos syriacus*	Bulgaria	EU665490	Davaineidae
Raillietina sp.*3*	*Picoides pubescens*	USA	EU665489	Davaineidae
Raillietina sp.*2*	*Picoides villosus*	USA	EU665488	Davaineidae
Raillietina tunetensis	*Leptotila verreauxi*	Costa Rica	EU665487	Davaineidae
Raillietina sp.*1*	*Crax rubra*	Costa Rica	EU665486	Davaineidae
Raillietina australis	*Dromaius novaehollandiae*	Australia	EU665484	Davaineidae
Taenia saginata	*Homo sapiens*	Kenya	AM503345	Taeniidae
Taenia hydatigena	Dog	China	HQ204207	Taeniidae
Taenia crassiceps	*Canis lupus*	France	JN849401	Taeniidae
Taenia regis	*Panthera leo*	Kenya	AM503348	Taeniidae
Hymenolepis diminuta	*Rattus rattus*	Spain	HM149291	Hymenolepididae
Hymenolepis sp.	*Microtus socialis*	Kazakhstan	HM149292	Hymenolepididae
Diphyllobothrium nihonkaiense[b]	*Canis lupus*	Canada	HQ423296	Diphyllobothriidae

[a]Query sequence generated.
[b]Used as out group.

RESULTS

LM and SEM observations

Four species of *Raillietina* subjected in this study included *R. echinobothrida*, *R. tetragona*, *R. cesticillus*, and *Raillietina* sp. (a species undetermined). They were morphologically different (Figs. 1-5) which were summarized in Table 3.

Sequence analysis

The length of ITS2 regions were varied among *Raillietina* spp. In the case of ND1 gene, sequence derived from *Raillietina* spp. were the same in length. Our *Raillietina* ITS2 and ND1 sequences were aligned with other *Raillietina* species from GenBank and then were trimmed to provide an equivalent sequence. The result revealed several insertions and deletions in the 748 bp alignment of ITS2 sequence among *Raillietina* spp. For the ND1 alignment revealed 213 variable nucleotide sites (no gab) in the length of 468 bp. These variable sites including 45 purine transitions, 26 pyrimidine transitions, 66 transversions and 76 multiple changes.

Table 3. Comparative morphology of *Raillietina* spp. found in domestic chickens in Thailand

	Raillietina echinobothrida	*Raillietina tetragona*	*Raillietina cesticillus*	*Raillietina* sp.
LM observation				
Scolex shape	round	ovoid	nearly round	round
Rostellum	armed	armed	armed	armed
Rostellar hook				
Row	2	1	2	2
Shape	hammer-shaped	hammer-shaped	hammer-shaped	hammer-shaped
Sucker shape	nearly round-shaped (armed)	ovoid-shaped (armed)	unarmed	round-shaped (armed)
genital pore opening	unilateral	unilateral	irregularly alternating	unilateral
Position of genital pore[a]	P	A	A	A
Number of egg/capsule	8-12	6-12	1	1
SEM observation				
Rostellar opening surface	scale-like spines	scale-like spines	smooth	scale-like spines
Strobila surface	pores	pores	pores	pores

[a]Position of genital pore: *P* = posterior to middle portion of each segment margin, A = anterior to middle portion of each segment margin.

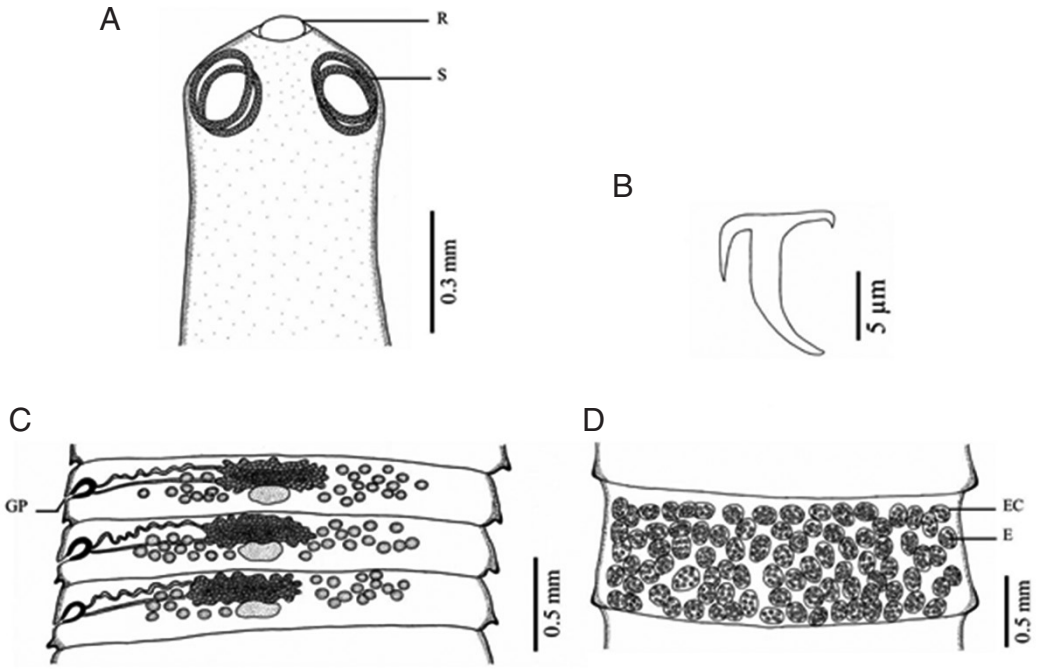

Fig. 1. Morphology of *Raillietina echinobothrida*. (A) Scolex bearing the rostellum (R) surrounded by 4 nearly round suckers (S). (B) Large hammer-shaped hook. (C) Mature proglottid showing unilateral opening of the genital pore (GP). (D) Gravid proglottid showing several eggs (E) per egg capsule (EC).

Phylogenetic relationships based on ITS2 region

The phylogenetic trees were reconstructed using NJ and ML methods (Fig. 6). The tree showed the most identical of tree topologies. This tree could be divided into 2 main clades; the first one was the helminth from order Cyclophyllidea, and the second one was the helminth from order Pseudophyllidea. The first clade was also divided into 2 subclades as the clade A that included all of *Raillietina* group, and the clade B that included the samples from the relative group. The *Raillietina* spp. group was monophyletic that had moderate bootstraps supported. *Raillietina* spp. were separated into 2 groups according to the number of eggs per egg capsule. *R. echinobothrida* and *R. tetragona* were grouped together with other *Raillietina* spp., while *R. cesticillus* and *Raillietina* sp. were in the same group.

Phylogenetic relationship based on ND1 gene

The phylogenetic trees were reconstructed using NJ and ML methods. The tree showed the most identical of tree topolo-

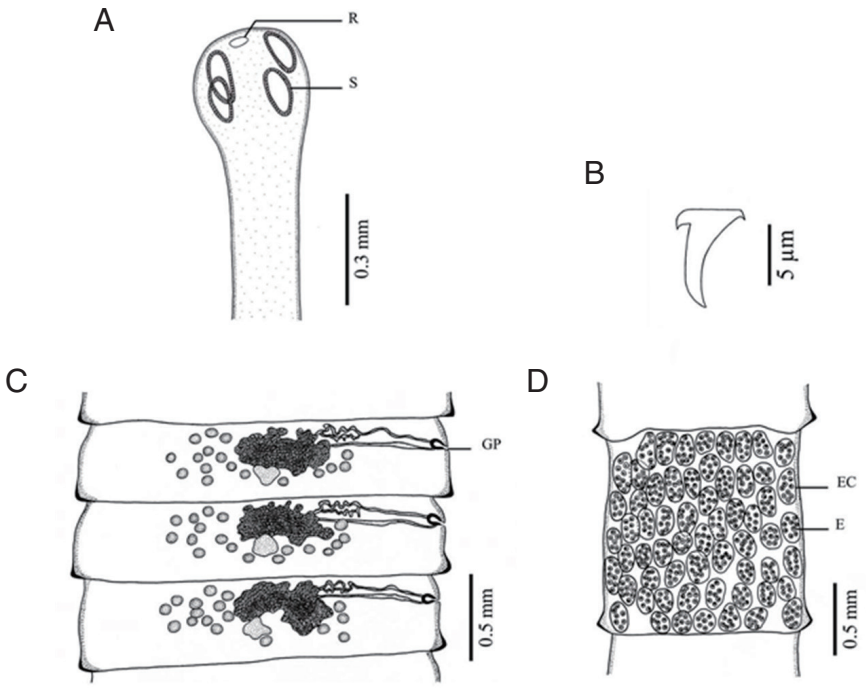

Fig. 2. Morphology of *Raillietina tetragona*. (A) Scolex bearing the rostellum (R) surrounded by 4 ovoid suckers (S). (B) Small hammer-shaped hook. (C) Mature proglottid showing unilateral opening of the genital pore (GP). (D) Gravid proglottid showing several eggs (E) per egg capsule (EC).

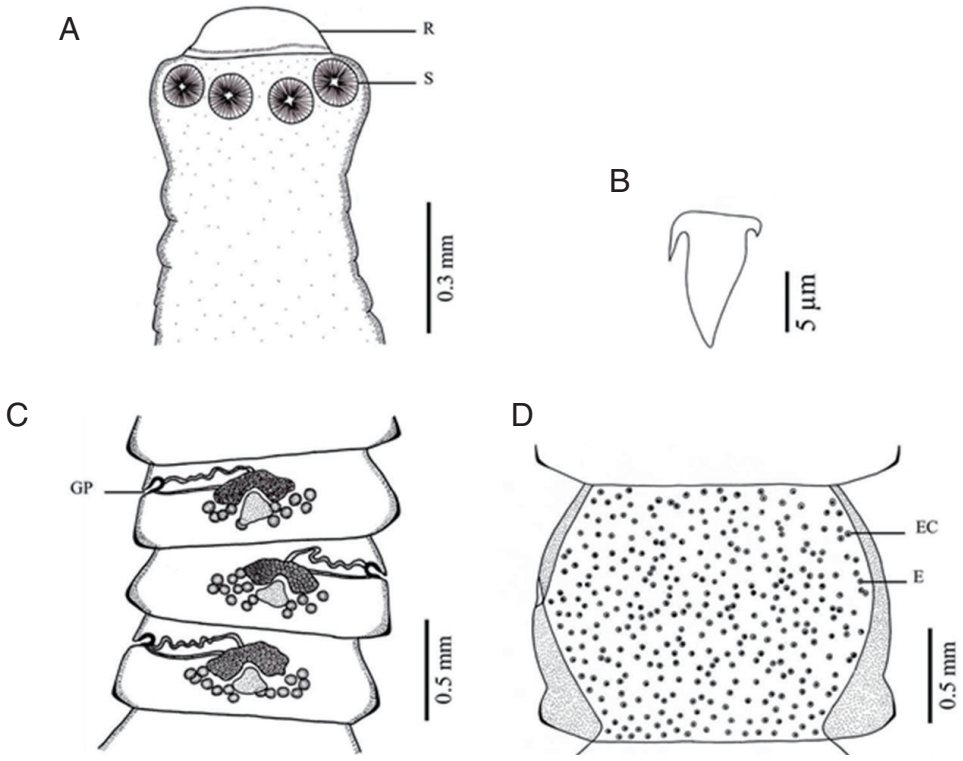

Fig. 3. Morphology of *Raillietina cesticillus*. (A) Scolex bearing the rostellum (R) surrounded by 4 unarmed round suckers (S). (B) Large hammer-shaped hook. (C) Mature proglottid showing irregularly alternating opening of the genital pore (GP). (D) Gravid proglottid showing a single egg (E) per egg capsule (EC).

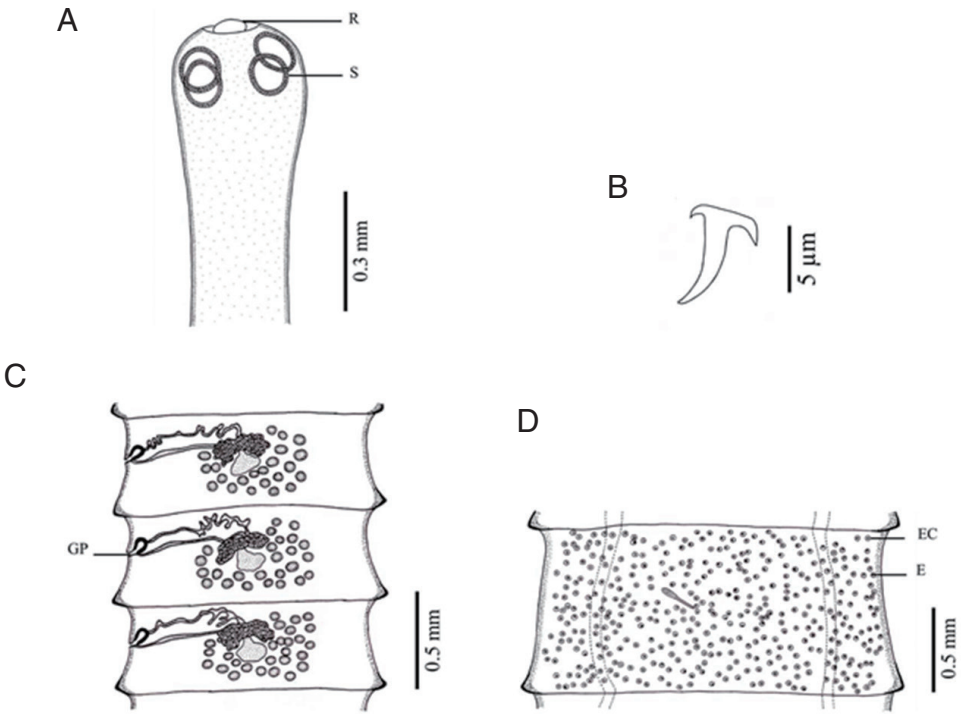

Fig. 4. Morphology of *Raillietina* sp. (A) Scolex bearing the rostellum (R) surrounded by 4 round suckers (S). (B) Small hammer-shaped hook. (C) Mature proglottid showing unilateral opening of the genital pore (GP). (D) Gravid proglottid showing a single egg (E) per egg capsule (EC).

gies as shown in Fig. 7. This tree could be divided into 2 main clades; the first clade was also divided into 2 subclades that were similar to the tree based on ITS2. The *Raillietina* group was monophyletic that had moderate bootstraps supported, and was separated into 3 groups according to unilateral and irregularly alternating openings of the genital pore and the number of eggs per egg capsule. These 3 groups included *Raillietina* sp. group, *R. cesticillus* group, and *R. echinobothrida* and *R. tetragona* which were grouped together with other *Raillietina* spp.

DISCUSSION

Based on LM observations, the morphological characters of *Raillietina* spp. in our study were similar to those of Hofstad et al. [4], Sawada [12], and Sawada [13]. The morphology of 4 *Raillietina* species was clearly different. The main features separating the species are the morphology of the scolex which differs in size and shape of the rostellum and suckers. *R. echinobothrida* and *Raillietina* sp. have round rostellum and nearly round and round suckers, respectively, while *R. tetragona* has small round rostellum and ovoid suckers, and *R. cesticillus* has large rostellum and suckers unarmed [4,12,13]. The rostellar hooks of *R. tetragona* are arranged in a single row while those of the 3 remaining species are arranged in 2 rows. Additionally, there are some more details revealing morphological dissimilarity. In *R. echinobothrida*, *R. tetragona*, and *Raillietina* sp., the genital pore is unilateral and situated in the posterior (*R. echinobothrida*), and anterior (*R. tetragona*, and *Raillietina* sp.) third portions of each segment, while in *R. cesticillus* it is irregularly alternating and situated in the anterior third portion of each segment. Moreover, *R. echinobothrida* and *R. tetragona* have several eggs per egg capsule within the gravid proglottid (8-12 and 6-12 eggs, respectively), whereas *R. cesticillus* and *Raillietina* sp. have a single egg per egg capsule. SEM observations showed the surface morphology of recovered *Raillietina* that was similar to previous studies [14-16]. The results revealed several pores on the body surface in all 4 species but different in the rostellar opening. *R. echinobothrida*, *R. tetragona*, and *Raillietina* sp. have scale-like spines along the edge of the opening, and only in *R. cesticillus* the edge is smooth.

Phylogenetic relationships of *Raillietina* spp. in domestic chickens from Thailand had not been studied before. The ribosomal DNA and mitochondrial genes have been widely used to identify and study phylogenetic relationships because

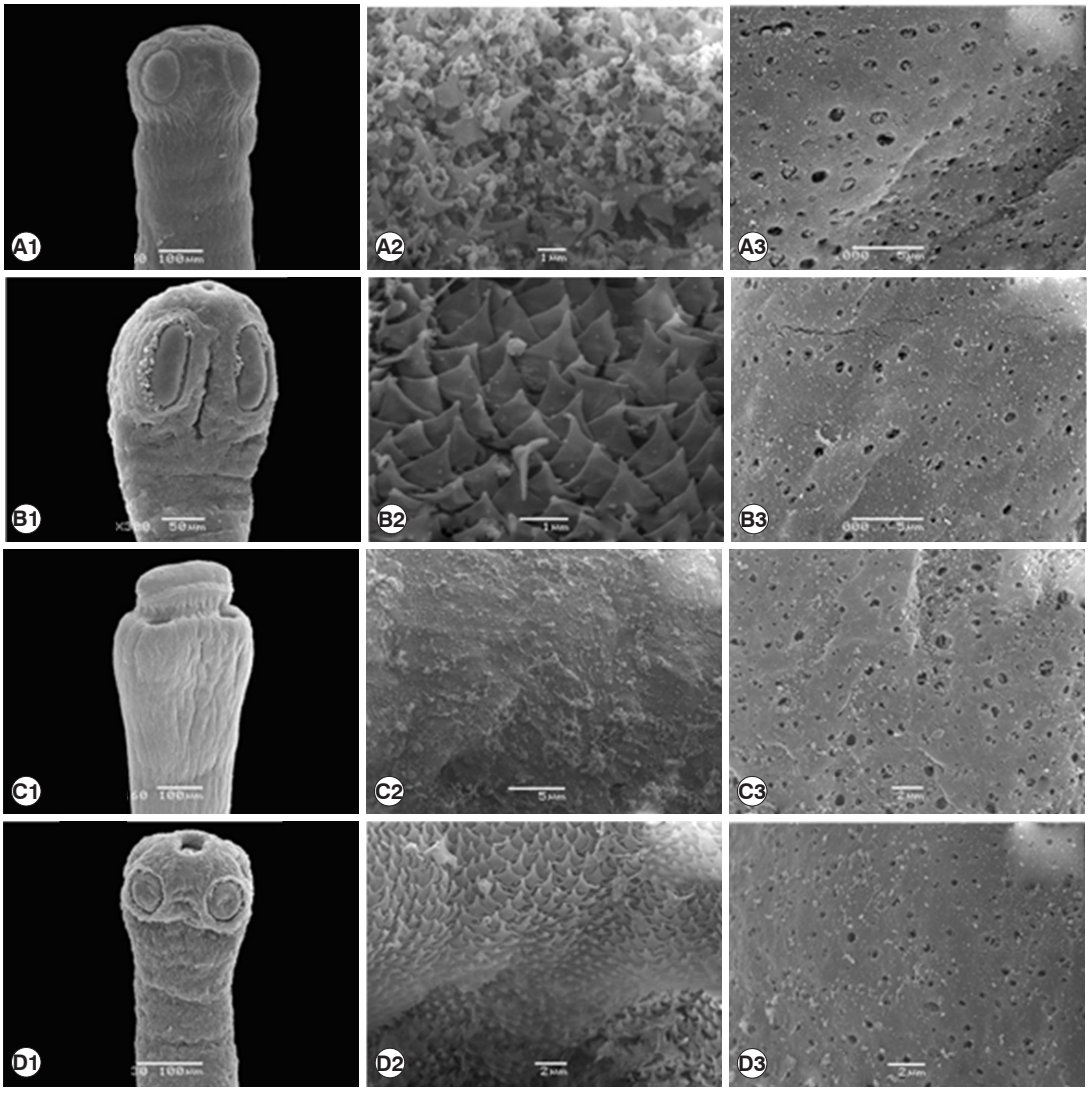

Fig. 5. SEM micrographs of 4 *Raillietina* species. (A) *R. echinobothrida*. (B) *R. tetragona*. (C) *R. cesticillus*. (D) *Raillietina* sp. (A1, B1, C1, D1) Scolex bearing the apical rostellum surrounded by 4 suckers. (A2, B2, D2) Rostellar opening revealing several scale-like spines along the edge. (C2) Rostellar opening revealing the smooth surface. (A3, B3, C3, D3) Strobila showing numerous pores on the surface.

they have fast evolutionary rates [17-19]. In our study, ITS2 and ND1 regions were successfully developed for studying the phylogenetic relationships of *Raillietina* spp. The results revealed that the ITS2 and ND1 sequence data recorded from *Raillietina* spp. appeared to be monophyletic. The bootstrapping of both trees indicated significant supports for grouping. These results were similar to previous studies [7,20]. The query sequences of *R. echinobothrida*, *R. tetragona*, *R. cesticillus*, and *Raillietina* sp. were aligned and placed within the Davaineidae group with other species of *Raillietina*, and separated from the Hymenolepididae and Taeniidae as a relative group, and also separated from the Diphyllobothridae as an out-group.

The phylogenetic trees derived from both ITS2 and ND1 sequence showed that each *Raillietina* was separated in correlation with the morphological characters and their definitive host. The phylogenetic relationships obtained using ITS2 sequences and the gravid proglottid characters could separate them into 2 groups. *R. echinobothrida* and *R. tetragona* were grouped together and closely related to other *Raillietina* species because of similarity in the egg number per egg capsule, i.e., several eggs in each capsule. *R. cesticillus* and *Raillietina* sp. had a single egg in each capsule and were grouped together. The phylogenetic relationships obtained from ND1 sequence showed a phylogenetic tree which differs from that based on

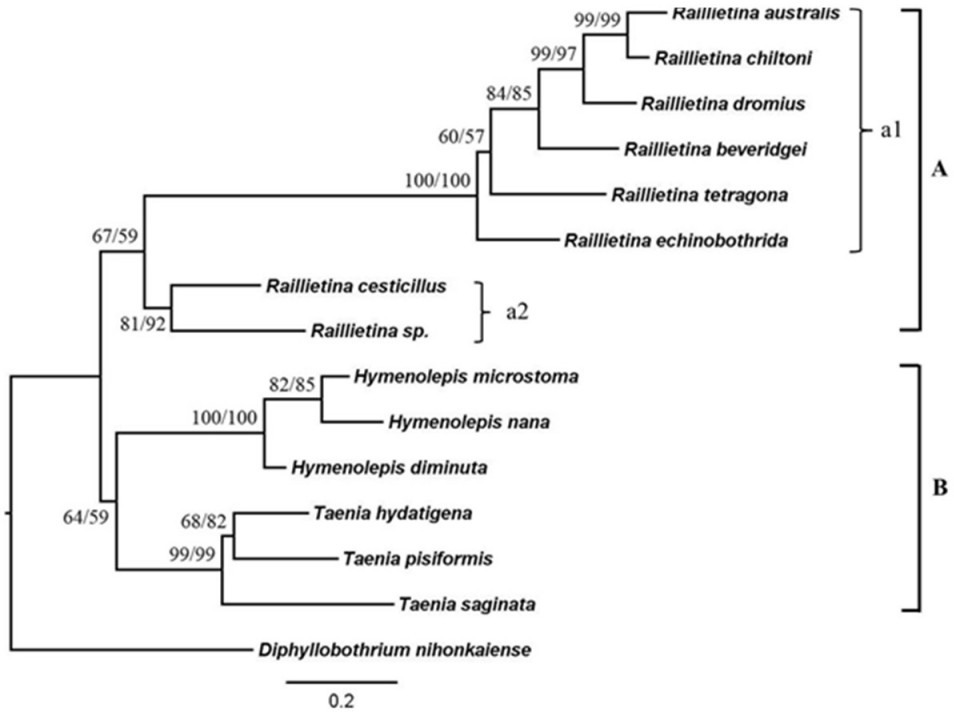

Fig. 6. Neighbor-joining (NJ) and maximum-likelihood (ML) bootstrap consensus tree with 1,000 bootstrap replicates of ITS2.

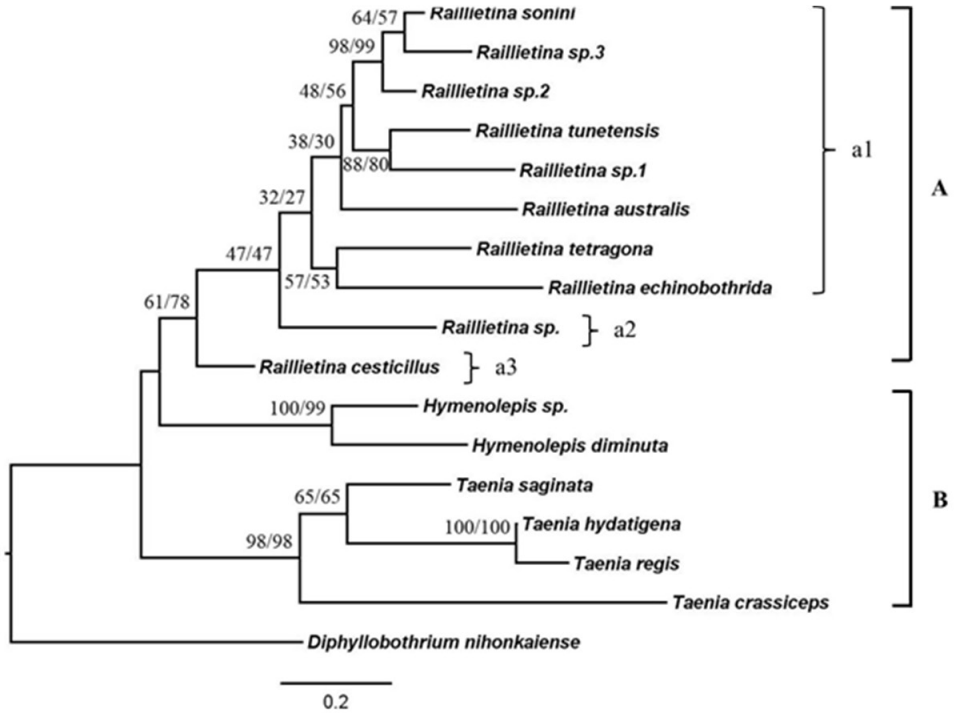

Fig. 7. Neighbor-joining (NJ) and maximum-likelihood (ML) bootstrap consensus tree with 1,000 bootstrap replicates of ND1.

ITS2 sequence. Each *Raillietina* species was separated according to the genital pore opening and the egg numbers in each egg capsule. The first group revealed unilateral genital opening and several eggs in each capsule, and included *R. echinobothrida, R.*

tetragona, and other *Raillietina* species. The second group had unilateral genital opening and a single egg in each capsule, and included *Raillietina* sp., and another group which had an irregularly alternating opening and a single egg in each egg capsule, and included *R. cesticillus*. *Raillietina* sp. was separated from *R. cesticillus* because of the different genital pore opening, while it was grouped to be a sister taxa of the first group because they have some similar morphologies, including suckers armed, unilateral genital opening, and scale-like spines along the rim of the rostellar opening. Moreover, each *Raillietina* in 'a1' group of ITS2 and ND1 trees was separated according to their definitive host. For example, *R. echinobothrida* and *R. tetragona* are more related to each other than the other *Raillietina* spp. because their definitive host is domestic chickens whereas the other *Raillietina* spp. use other birds as their definitive host [2,8,21].

Our study can conclude that the sequence data of ITS2 and ND1 regions combined with morphological observations using LM and SEM can be a useful tool for identification and understanding the phylogenetic relationships in the *Raillietina* group. Their relationships were defined according to the systematic criteria. Furthermore, the phylogenetic tree derived from ND1 sequence could separate the different morphologies of the genital pore opening (unilateral and irregularly alternating) because the evolution of the mitochondrial gene is faster than the nuclear gene.

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CONFLICT OF INTEREST

We have no conflict of interest related to this study.

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