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Research article

Hematological and phylogenetic studies of *Leucocytozoon* spp. in backyard chickens and fighting cocks around Kamphaeng Saen, Thailand

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Abstract

Leucocytozoon infections are found in birds and chicken in Southeast Asia including Thailand. Although chicken *Leucocytozoon* infections are common in Thailand, the associated hematological and molecular studies are scarce. Blood samples were collected from 52 backyard birds (22 chickens, 16 fighting cocks (FCs), 13 ducks and 1 goose) from four provinces (Ayutthaya, Nakhon Pathom, Pathum Thani and Kanchanaburi) in Thailand and processed for complete blood cell counts. Polymerase chain reaction (PCR) was performed for a partial cytochrome *b* gene from *Leucocytozoon*. Microscopic examinations revealed that 8 chickens and 13 ducks (21/52, 40%) were negative for blood parasites but 9 chickens and 15 FCs (24/52, 46%) were positive for *Leucocytozoon* spp. Three chickens and 7 FCs had mixed infections with microfilaria, *Plasmodium*/*Haemoproteus* spp. and *Trypanosoma* spp. There were no significant differences in the extensive hematological values among low rate (< 0.01% *Leucocytozoon* parasites), high rate (> 0.01% *Leucocytozoon* parasites) and mixed infections, except for the packed cell volume, hemoglobin concentration and fibrinogen concentration that were lower and for punctate reticulocytes that were higher in the high rate infections ($p < 0.05$). The information based on gametocytes and their host cells identified *L. sabrasezi*. In total, 24 PCR positive samples (both chickens and FCs) were divided into five groups based on four positions of 462 base pair amplicons without primer regions. Phylogenetic analysis showed that groups I–III were 99.1–99.8% similar and also closely related (98.0% similarity) to leucocytozoids isolated from chicken in Malaysia. Microscopy and molecular studies revealed similar morphology and phylogenetic groups of *Leucocytozoon* in both chickens and FCs.

Introduction

Leucocytozoids (Apicomplexa, Haemosporida, Leucocytozoidae) are avian blood parasites which infect numerous species of avian hosts (Valkiūnas, 2005; Forrester and Greiner, 2009; Valkiūnas et al., 2010; Zhao et al., 2014). The pathogenicity of all leucocytozoids is host-specific at the avian level of order, family and in some cases at the species level (Forrester and Greiner, 2009). *Leucocytozoon* in Thailand have been reported in 1976 in *Zoothera marginata* Blyth (Greiner, 1976). *Leucocytozoon* has commonly occurred in domestic chickens (*Gallus gallus domesticus*, Galliformes) from Thailand (Worasing et al., 2001; Tongkamsai and Napoon, 2015; Takang et al., 2017) and other countries in Southeast Asia (Paperna et al., 2008). Based on the current taxonomy, three species of *Leucocytozoon*

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were found in domestic chickens—*L. macleani* Sambon, 1908; *L. caulleryi* Mathis and Léger, 1909 and *L. schoutedeni* Rodhain, Pons, Vandenbranden and Bequaert, 1913 (Valkiūnas, 2005; Forrester and Greiner, 2009). In particular, (*Akiba*) *L. caulleryi* and *L. macleani* (synnoname *L. sabrazesi*) have appeared in chickens of Thailand for a long time (Bennett et al., 1991; Worasing et al., 2001; Tongkamsai and Napoon, 2015; Takang et al., 2017). *Leucocytozoon schoutedeni* and *L. macleani* are much less pathogenic than *L. caulleryi* (Valkiūnas, 2005). *L. simondi* has been reported in ducks and geese from Vietnam (Valkiūnas, 2005).

Infections by *Leucocytozoon* spp. (leucocytozooniasis) are diagnosed by examining stained thin blood smears and finding the characteristic gametocytes (Valkiūnas, 2005; Forrester and Greiner, 2009). The monograph by Valkiūnas (2005) contained pertinent information as a very useful resource for the identification of leucocytoziods. Molecular polymerase chain reaction (PCR) diagnostic methods were developed in several genes of *Leucocytozoon* spp. A cytochrome *b* gene (cyt *b*) (Hellgren et al., 2004; Bernotienė et al., 2016) was the most commonly used gene, because it provides a slower molecular clock for these parasites than for their host cells (Bensch et al., 2013). Therefore, numerous data regarding this gene have been stored in Genbank and MalAvi (Bensch et al., 2009; Valkiūnas et al., 2010). Recent molecular work using the cyt *b* gene has shown *Leucocytozoon* parasites to be highly diverse (Sehgal et al., 2006a; Valkiūnas et al., 2010). All molecular diagnostic and microscopic examinations have their differences as well as their advantages and disadvantages. For example, leucocytozoids have been identified by linking molecular tests with traditional morphological species (Martinsen et al., 2006). Notably, the combination of a molecular technique and microscopic examination was the best choice for diagnosis of *Leucocytozoon* and other avian haemosporidians (Cosgrove et al., 2006; Valkiūnas et al., 2008; Garamszegi, 2010). However, the linkage of lineages (haptotypes) with conventional morphology to specifically identify *Leucocytozoon* is very scarce. Most found and described species of *Leucocytozoon* have remained unnamed (Paperna et al., 2008).

The high prevalence of *L. sabrazesi* infections has been reported in backyard chickens from northern Thailand (Jaijan et al., 2012; Takang et al., 2017). Although, chicken *Leucocytozoon* infections were common in Thailand (Bennett et al., 1991; Worasing et al., 2001; Tongkamsai and Napoon, 2015; Takang et al., 2017), studies on their hematology and molecular makeup in Thailand are scarce. Thus, the current research combined microscopic and molecular techniques to identify *Leucocytozoon* relationships between backyard fighting cocks (FCs) and chickens in four central Thai provinces. In addition, variability of hematological values of leucocytozooniasis in each group was also discussed. Ducks and a goose raised on the same farms were also included in this study.

Materials and Methods

Ethics statements

The present study was approved by the Institutional Laboratory Animal Care and Use Committee of Kasetsart University, Thailand under protocol number ACKU 01560. All applicable international, national and/or institutional guidelines for the care and use of animals were followed. There were no human participants in this study.

Blood collection

Blood samples were collected from 52 clinically healthy backyard birds (22 chickens, 16 FCs, 13 ducks and 1 goose; Table 1) from free-range farms in four central Thai provinces: Ayutthaya, (from three free-range farms, located at 14.3272820N, 100.5490600E; 14.3298490N, 100.5483490E; 14.3190700N, 100.5298410E), Nakhon Pathom (14.0168610N, 99.9749730E), Pathum Thani (14.0136080N, 100.4136160E) and Kanchanaburi (14.3605431N, 99.6791365E), from May to December 2016. More than 700 µL whole blood was obtained from the jugular or brachial vein or wing veins and then gently transferred to a tube containing ethylene diamine-tetra acetic acid (Salakij et al., 2015).

The blood samples were analyzed for a complete blood cell count (CBC or hematology) using the same procedure as described previously (Salakij et al., 2015). The total solids concentration was determined using a refractometer. Blood smears were stained using Wright's stain [in house preparation using, Wright eosin methylene blue and Giemsa's azure eosin methylene blue (Merck KGaA; Darmstadt, Germany)]. A BX53 light microscope (Olympus; Tokyo, Japan) equipped with a DP73 digital camera and CellSense Standard (Olympus imaging software, Tokyo, Japan) was used to examine the blood smears, to take the photographs and to measure the parasites. The intensity of parasitemia was estimated as a percentage by actual counting of the number of parasites per 10,000 red blood cells (Valkiūnas et al., 2014). The infection intensity was divided into low grade (less than 0.01% *Leucocytozoon* parasites) and high grade (more than 0.01% *Leucocytozoon* parasites). The combination of other blood parasites and *Leucocytozoon* infections were grouped as mixed infection.

Table 1 *Leucocytozoon* (*L.*) and other blood parasites (BP) infection rates (number of birds) in studied birds

Avian species	BP negative	*L.* spp.	*L.* spp. and other BPs	Total *L.* infection rates	Other BPs
Chickens	36% (8/22)	27% (6/22)	14% (3/22)[1]	41% (9/22)	23% (5/22)[3]
Fighting cocks	-	50% (8/16)	44% (7/16)[2]	94% (15/16)	6% (1/16)[4]
Ducks	100% (13/13)	-	-	0	-
Goose	-	-	-	0	100% (1/1)[‡]
Total	40% (21/52)	27% (14/52)	19% (10/52)	46% (24/52)	14% (7/52)

[1] Mixed infections with microfilaria; [2] Mixed infections with microfilaria (n = 1), *Plamodium* spp. (n = 1), trypanosome (n = 2), and microfilaria and *Plasmodium* spp. (n = 3); [3] Infected with *Plasmodium* spp.; [4] Infected with microfilaria

The *Leucocytozoon*-positive blood samples occurred in the two chicken farms located in Pakkarn (PK), Ayutthaya province and on a free-range farm in Kamphaeng Saen (KPS), Nakhon Pathom province. All 24 samples that were *Leucocytozoon* positive were divided into three groups based on the type of host: FCs in Pakkran subdistrict (PK01, 02, 04–10, 14, $n = 10$), FCs (KPS1, 3, 4, 9, 10F, $n = 5$) and chickens (KPS5-8, 11–15C, $n = 9$) in Kamphaeng Saen district.

DNA Extraction, polymerase chain reaction amplification and sequencing

DNA extraction from each of the 52 blood samples was performed using a Favorgen prep blood DNA extraction mini kit (Favorprep; Taiwan). Nested PCR amplified cyt *b* gene for detecting *Leucocytozoon* was used following the protocol described by Hellgren et al. (2004). Briefly, the first primer set was the Haem NF1 (5'-CATATATTAAGAGATTATGGAG-3') and Haem NR3 (5'-ATAGAAAGATAAGAATACCATT-3') primers. The nested primer used Haem FL (5'-ATGGTGTTTTAGATACTTACATT-3') and HaemR2L (5'-CATTATCTGGATGAGATAATGGIGC-3. The first PCR reaction was done in 20 µL of each sample, consisting of 0.5 µL Dream taq, dNTP, primers, $MgCl_2$ 50 mMol and DNAese free water (Thermo Fisher Scientific; Thailand). The first PCR reaction was run according to the protocol: initial denaturation at 95°C for 3 min, denaturation at 95°C for 30 sec, annealing at 52°C for 30 sec, extension at 72°C for 45 sec and final extension at 72°C for 10 min (35 cycles). The second PCR reaction was done in the same volume and using the same protocol for the PCR reaction. For the second reaction, 2 µL of 10-fold dilution of the first reaction was used as a template. A 2 µL sample of the second PCR product was subjected to 1.5% agarose gel electrophoresis. One negative control (nuclease free water) and one positive control (microscopy *Leucocytozoon* positive blood sample) was used to prevent contamination or false amplification. The amplicons were stained with Gel red™ (Biotium, Thailand). The second PCR product was purified using a Favorprep Gel/prep Purification mini kit (Favorgen Biotech; Taiwan) and sequenced by First BASE Ltd. (Selangor, Malaysia). Molecular study of *Plasmodium*/*Haemoproteus* was not undertaken.

For detecting trypanosome infections, all birds were screened using nested PCRs as reported by Pornpanom et al. (2019). Briefly, total DNA was extracted from 50 µL of each blood sample, using a Blood Genomic DNA Extraction kit (FavorPrep™; Favorgen Biotech Corp.; Taiwan). The SSU rRNA gene fragment was amplified by the nested PCR (Valkiūnas et al., 2011). The primary reaction used 2 µL of each extracted DNA sample in DreamTaq Green PCR Master Mix (2X) (ThermoScientific; USA) and 10 nMol primers (Tryp763 and Tryp1016). The primary products were diluted with 10-fold RNase-free water and 2 µL aliquots of those solutions were used as secondary templates. The primers for the secondary templates were Tryp99 and Tryp957. Amplicons (770 base pairs; bp) of the SSU rRNA gene were stained with fluorescent dye (GelRed® nucleic acid gel stain; Biotium Inc.; Fremont, CA, USA) and electrophoresed on a 1.5% agarose gel at 132 V for 20 min. There were no template controls or positive controls in any of the reactions used to avoid false reactions.

Phylogenetic analysis

The phylogenetic analysis was based on sequences (456 bp) of the cyt *b* gene contributed by 41 sequences of *Leucocytozoon* and one outgroup of *Plasmodium circumflexum* KU81 (JN639001) in a shikra from Thailand. All samples were separated into 18 sequences of *Leucocytozoon* in chicken and FCs (PK, KPS, respectively) and the remaining 23 sequences were obtained from GenBank. The evolutionary distances were computed using the Tamura 3-parameter model (Tamura, 1992). The analysis involved 30 nucleotide sequences. All positions containing gaps and missing data were eliminated. In total, there were 151 positions in the final dataset. Evolutionary analyses were conducted using the MEGA7 software package (Kumar et al., 2016).

The tree topology for *Trypanosoma* sp. from one FC was generated containing the one small subunit ribosomal RNA (SSU rRNA) sequence from the current sample and 48 published sequences (Zídková et al., 2012) deposited in GenBank. Two sequences of trypanosomes infecting non avian hosts were used as out groups, namely AJ009161 (*T. rotarium* B2-II) and AJ009157 (*T. mega* ATCC30038). All sequences were the same length (798 bp). The best-fit model was the Kimura 2-parameter model with gamma distribution (Kimura, 1980); maximum-likelihood phylogeny with 1,000 bootstrap replicates was implemented using the MEGA 7 software package (Kumar et al., 2016).

Statistical analysis

Hematological results among three groups (low, high single *Leucocytozoon* infection and mixed infection) were presented as mean ± SD. The Kruskal-Wallis multiple-comparison Z-value test and the Bonferroni test were used to determine statistical significance among different blood parasite infection groups with a p value < 0.05 defined as significant. All statistical analyses were performed using the NCSS 2007 software package (Hintz; Kaysville, UT, USA).

Results

Blood parasite morphology

Microscopic examination of blood parasites revealed that 8 chickens and 13 ducks were negative for blood parasites but 9 chickens and 15 FCs (24/52, 46.2%) were positive for only *Leucocytozoon* (Table 1). Both macro- and micro-elongate gametocytes were found in both FCs (Figs. 1A and 1B) and chickens (Figs. 1E and 1H) and had similar shapes. Elongate gametocytes displaced the host cell nucleus with a gametocyte like a cap or bean-shaped but never dumbbell-shaped. The host cell cytoplasmic process contained a few small vacuoles with volatile granules usually presented, having a wide base and long tail. Elongate microgametocytes were larger than the macrogametocytes. The cytoplasm of macrogametocytes stained deep blue and contained variable sizes of vacuoles (Figs. 1A and 1E). Microgametocytes were pale blue with a prominent pink nucleus

(Figs. 1B and 1F). The macro- and micro-roundish gametocytes were roundish- or oval-shaped, similar to the nucleus of the parasite (Figs. 1C, 1D, 1G and 1H). The host cell nucleus was ring-shaped, with variable length, with more than half the circumference of the gametocytes. Based on the information on gametocytes and their host cells, the *Leucocytozoon* samples in both FCs and chickens were identified as *L. sabrasezi* (Valkiūnas, 2005).

Of the nine *Leucocytozoon*-positive birds, three chickens were mixed infections with microfilaria and six FCs were mixed infections with *Plasmodium/Haemoproteus* spp. (n = 1, Fig. 2A), trypanosome (n = 2, Fig. 2B), microfilaria (n = 1, Fig. 2C) and microfilaria and *Plasmodium* (n = 3, Table 1). Only *Plasmodium* spp. was found in five chickens and the one goose and microfilaria were found in one FC (Table 1). The total *Leucocytozoon* infection rate was higher in FCs (94%) than in chickens (41%; Table 1).

Hematology

Comparative hematology between the three groups of leucocytozooniasis (from both chicken and FCs) revealed that there was few significant differences among low grade infections (less than 0.01% *Leucocytozoon* parasites, n = 5), high grade infections (more than 0.01%, n = 9) and leucocytozooniasis with mixed infection with other blood parasites (n = 10, Table 2). The exceptions were the packed cell volume (PCV), hemoglobin concentration and fibrinogen concentration in the high grade infection group that were lower than those of the other two groups. The low grade infections also had a lower punctate reticulocyte number than those of high grade infection. The hematological values in leucocytozooniasis with mixed infection with other blood parasites were not significantly different ($p > 0.05$) from both the low and high grade only *Leucocytozoon* infections (Table 2).

Sequences analysis

In total, 24 polymerase chain reaction-positive samples (accession numbers KX950720–KX950743) (from both chickens and FCs) were divided into five groups based on four positions of the 462 bp amplicon without primer regions, consisting of the 3768, 3879, 4044 and 4055 positions which were referred to the *L. fringillinarum* mitochondrion complete genome (NC012451). The majority of positive samples (14 samples) were grouped as group I which consisted of nine samples from Pakkran, Ayutthaya province (PK1F, PK2F, PK4F, PK5F, PK7F–PK10F, PK14F) and five samples (KPS04F, KPS05F, KPS11C, KPS12C, KPS13C) from Kamphaeng Sean, Nakhon Pathom province. There were two samples in both group II (KPS8C, KPS9F) and group III (KPS6C, KPS7C), as shown in Table 3.

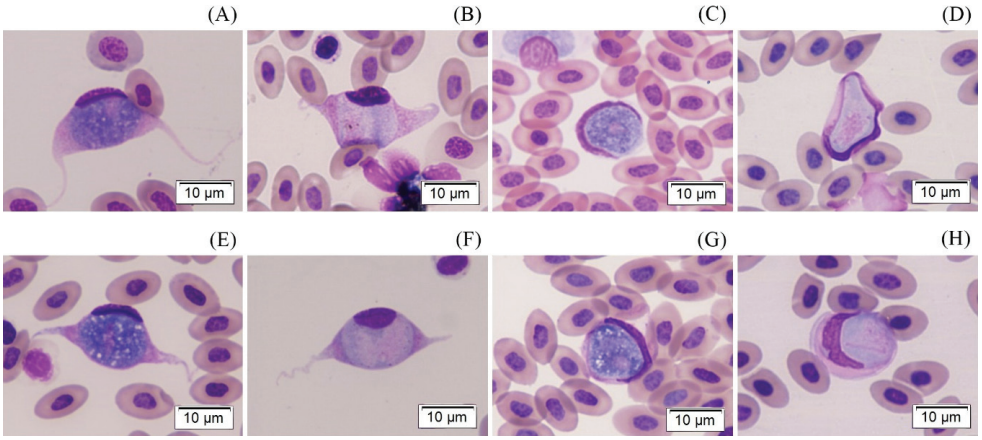

Fig. 1 Light micrographs of *Leucocytozoon* spp. in fighting cocks: (A–D) and backyard chickens in Kamphaeng Saen (E–H): (A) and (E) elongate macrogametocytes; (B) and (F) elongate microgametocytes; (C) and (G) roundish macrogametocytes; (D); (H) roundish microgametocytes, all using Wright's stain

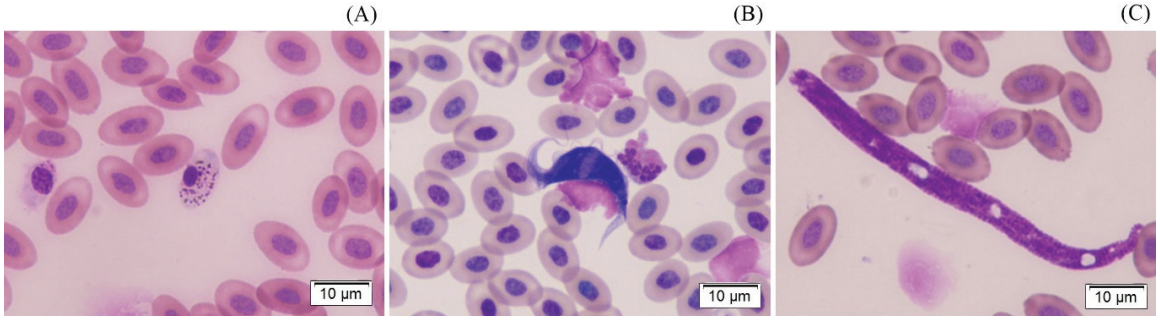

Fig. 2 Light micrographs of other blood parasites co-infected with *Leucocytozoon* spp. in chickens and fighting cocks: (A) *Plasmodium* sp.; (B) *Trypnosoma avium*; (C) microfilaria, all using Wright's stain

Table 2 Comparative hematology (mean ± SD) of chicken leucocytozooniasis (both chicken and fighting cocks) depending on infection rates and other blood parasite (BP) infections

Parameter	Leucocytozooniasis depending on infection rate		Leucocytozooniasis and other BP infections
	< 0.01%	> 0.01%	
Number of chickens	5	9	10
Packed cell volume (%)	38.40 ± 1.39[a]	31.44 ± 1.00[b]	36.00 ± 1.60[ab]
Red blood cells count ($\times 10^{12}$/L)	3.38 ± 0.12	2.98 ± 0.16	3.05 ± 0.15
Hemoglobin (g/L)	110.62 ± 4.40[a]	92.03 ± 2.80[b]	100.00 ± 0.50[ab]
Mean corpuscular volume (fL)	115.06 ± 3.80	107.41 ± 5.33	116.00 ± 4.80
Mean corpuscular hemoglobin (pg)	32.78 ± 1.06	31.61 ± 1.72	33.00 ± 1.70
Mean corpuscular hemoglobin concentration (g/dL)	28.61 ± 1.30	29.38 ± 0.23	29.20 ± 7.40
White blood cells count ($\times 10^{9}$/L)	13.96 ± 1.69	10.54 ± 1.07	17.03 ± 3.42
Heterophils	3.82 ± 0.37	3.93 ± 0.79	6.42 ± 1.72
Eosinophils	0.89 ± 0.24	0.57 ± 0.15	1.00 ± 0.23
Basophils	0.56 ± 0.17	0.47 ± 0.07	0.51 ± 0.08
Lymphocytes	7.19 ± 1.05	4.42 ± 0.65	1.67 ± 1.45
Monocytes	1.50 ± 0.25	1.15 ± 0.25	1.43 ± 0.31
Differential count (%)			
Heterophils	28.00 ± 1.79	36.78 ± 5.25	36.45 ± 2.80
Eosinophils	6.00 ± 1.14	5.89 ± 1.70	6.00 ± 1.20
Basophils	4.00 ± 1.05	4.44 ± 0.44	3.00 ± 0.50
Lymphocytes	50.80 ± 2.06	42.56 ± 4.36	45.50 ± 4.10
Monocytes	11.20 ± 1.85	10.33 ± 1.55	8.45 ± 0.90
Heterophil:Lymphocyte ratio	0.56 ± 0.06	1.07 ± 0.26	0.90 ± 0.10
Thrombocytes (%WBC)	180.80 ± 16.99	190.11 ± 19.72	156.00 ± 16.80
Reticulocytes			
Aggregate (%RBC)	2.90 ± 0.97	3.42 ± 1.00	2.93 ± 0.90
Punctate (%RBC)	21.36 ± 5.00[a]	59.62 ± 1.97[b]	41.92 ± 6.80[ab]
Plasma protein (g/L)	50.04 ± 2.14	48.00 ± 2.03	49.00 ± 2.30
Fibrinogen (g/L)	5.20 ± 1.02[a]	2.00 ± 0.53[b]	3.20 ± 0.88[ab]

WBC = white blood cell; RBC blood cell
mean values with different lowercase superscripts within each row are significantly different ($p < 0.05$).

Table 3 Summary of code name, type of avian host, locality, blood parasite infections (microscopic examination), GenBank accession numbers (Acc. No.), phylogenetic groups of 24 *Leucocytozoon* isolates and grading of *Leucocytozoon* infections

Code name	Type of host	Locality	Type of blood parasite	*Leucocytozoon* GenBank Acc. No.	Phylogenetic group	Grading of *Leucocytozoon* infection (%gamonts /10,000 RBCs)
PK01F	FC	PK	L	KX950720	I	< 0.01
PK02F	FC	PK	L, M, P/H	KX950721	I	< 0.01
PK04F	FC	PK	L, P/H	KX950722	I	< 0.01
PK05F	FC	PK	L	KX950723	I	< 0.01
PK07F	FC	PK	L	KX950725	I	< 0.01
PK08F	FC	PK	L, M	KX950726	I	< 0.01
PK09F	FC	PK	L, P/H, M	KX950727	I	< 0.01
PK10F	FC	PK	L	KX950728	I	< 0.01
PK14F	FC	PK	L	KX950729	I	< 0.01
KPS04F	FC	KPS	L, T	KX950732	I	< 0.01
KPS05C	BC	KPS	L	KX950733	I	> 0.01
KPS11C	BC	KPS	L, M	KX950739	I	> 0.01
KPS12C	BC	KPS	L	KX950740	I	> 0.01
KPS13C	BC	KPS	L, M	KX950741	I	> 0.01
KPS08C	BC	KPS	L	KX950736	II	> 0.01
KPS09F	FC	KPS	L	KX950737	II	> 0.01
KPS06C	BC	KPS	L	KX950734	III	> 0.01
KPS07C	BC	KPS	L	KX950735	III	> 0.01
PK06F	FC	PK	L, M, P/H	KX950724	IV	> 0.01
KPS01F	FC	KPS	L, T	KX950730	IV	> 0.01
KPS03F	FC	KPS	L	KX950731	IV	> 0.01
KPS15C	BC	KPS	L	KX950743	IV	> 0.01
KPS10F	FC	KPS	L	KX950738	V	> 0.01
KPS14C	BC	KPS	L, M	KX950742	V	> 0.01

FC = Fighting cock; BC = Backyard chicken; KPS = Khamphaeng Saen, Nakhon Pathom province; PK = Pakkarn, Ayutthaya province; < 0.01 = low infection rate; > 0.01 = high infection rate; < 0.01 and > 0.01 = mixed infections; L = *Leucocytozoon* spp.; M = microfilaria; P/H = *Plasmodium*/*Haemoproteus* spp.; T = trypanosome

Four samples of group IV (PK6F, KPS1F, KPS3F, KPS15C) presented double peaks on two positions of an electropherogram of nucleotide sequences; the first one was the combination of base A (alanine) or T (thymine), represented as W at position of 3876 cyt *b* nucleotide sequence and the other was the combination of base T or C (cytosine), represented as Y at position of 4044 cyt *b* nucleotide sequence. From four different positions of nucleotide sequences in group IV, true nucleotide sequences suggested the identical sequence of group I (TATC) and also three patterns of TACC, TTCC and TTTC (Supplementary Table 1). Group V contained two samples (KPS10F, KPS14C) isolated from one FC and one chicken. This group contained a degenerated sequence (G, guanine or C) on position 4055 of the cyt *b* nucleotide sequence which might be an identical nucleotide sequence in both group II (CTCC) and group III (CTCG). Position 4055 of the nucleotide sequence in group III was only a non-synonymous substitution that altered amino acid residue from A to G. Thus, only the 18 sequences in groups I–III were analyzed using a phylogenetic tree.

Table 3 summarizes the code name, type of avian host, locality, blood parasite infection (microscopic examination), GenBank accession numbers of the *Leucocytozoon* isolates, phylogenetic groups and grading of *Leucocytozoon* infection in each bird. High infection rates were identified in two of the four studied sites (Pakkarn in Ayutthaya province and Kamphaeng Sean in Nakhon Pathom province). Group I and group IV contained sequences from both sites while the remaining group was confined to Kamphaeng Sean in Nakhon Pathom province.

Phylogenetic analysis

Phylogenetic analysis (Fig. 3) using the neighbor-joining method among the three groups showed that group I was 99.3% and 99.1% similar to group II and group III, respectively. Group II and group III displayed 99.8% sequence similarity. All samples from the current study were closely related (98.0% similarity) with leucocytozoids isolated from *Gallus gallus* in Malaysia (KT290945 and KT290946). Furthermore, all groups of leucocytozoids were genetically divergent with *L. schoutedeni* from chicken (KT290936) by more than 3.9%; with *L. fringillinarum* from passerine (NC012451) by more than 8.4%; with *L. ziemanni* (synonym *L. danilewskyi*) from owl (EU624137) by more than 8.9% (8.9–9.1%) and with *L. sabrazesi* (KT290930) and *L. caulleryi* (NC015304) from chicken by more than 14% and 14.8%, respectively.

Comparative hematology between the three phylogenetic groups of leucocytozooniasis revealed no significant differences among groups. The phylogeny of the SSU rRNA sequence from the FC (KPS01F) revealed that the trypanosome species isolate was grouped together with other *T. avium* sequences with 100% similarity.

Discussion

The high blood parasite infection rate in both backyard chickens and FCs around Kamphaeng Saen, Nakhon Pathom province may have been due to the free-range habitats that could increase intermediate insect-host contacts. The infection rate of *L. sabrazesi* in the FCs (93.75%) was higher than for backyard chickens (662/856, 77.3%) in Chiang Mai, Thailand (Takang et al., 2017) and indigenous Thai chickens (306/446, 68.61%) in Nan province, Thailand (Jaijan et al., 2012) but as high as in three of seven sites in China (Zhao et al., 2014). It was also higher than the prevalence of *L. schoutedeni*-infected village chickens (18.3%) in Uganda and Cameroon (Sehgal et al., 2006b). These differences may have been due to geographical and seasonal differences. Co-infections are reportedly common in Thailand with other avian malaria or trypanosomes or both (Jaijan et al., 2012) or microfilaria or both (Takang et al., 2017). There was no *Leucocytozoon* infection in the ducks and the goose in the current study, though there was a report of *L. simondi* in ducks and geese from Vietnam (Valkiünas, 2005).

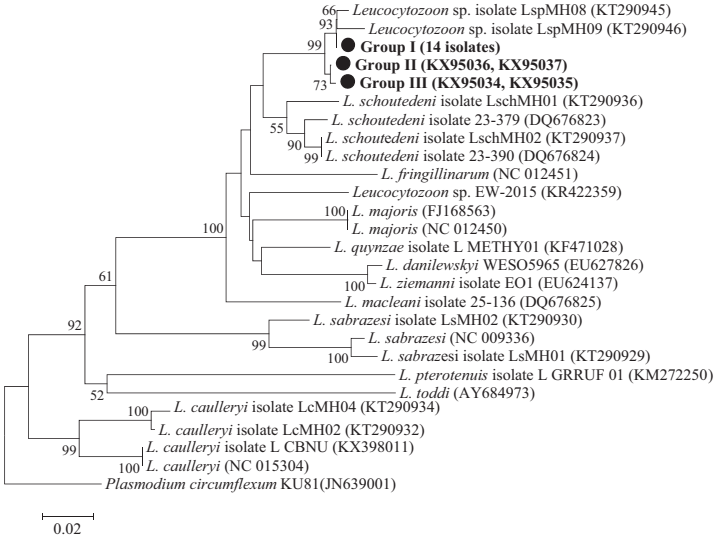

Fig. 3 Phylogenetic trees based on partial cytochrome *b* DNA sequences (462 base pairs) of 18 *Leucocytozoon* isolates (Groups I–III in bold) using neighbor-joining and Tajima-Nei methods, with 1,000 bootstrap replicates with the majority rule consensus tree was more than 50%, Genbank accession numbers are shown in the parentheses and *Plasmodium circumflexum* is a root of the tree.

Supplementary Table 1 Position of nucleotide sequence differences in cytochrome *b* gene of *Leucocytozoon* spp. in 9 chickens and 15 fighting cocks which was referred position of nucleotide sequence from *L. fringillinarum* (NC012451)

	Position of nucleotide sequence on cytochrome *b* gene				Number of chickens and fighting cocks	Code
	3768	3879	4044	4055		
Group I	T	A	T	C	14	TATC
Group II	C	T	C	C	2	CTCC
Group III	C	T	C	G	2	CTCG
Group IV	T	W*	Y*	C	4	TWYC
Group V	C	T	C	S*	2	CTCS

* W = Adenine or Thymine; Y = Thymine or Cytosine; S = Guanine or Cytosine.

The low PCV and hemoglobin concentration in the high rate *Leucocytozoon* infection indicate the destruction of erythrocytes by the leucocytozoids which also slightly increased the reticulocyte number in response of the anemia (Campbell, 2015). However, leucocytozoids in chickens did not appear to change the hematology profiles compared to the normal interval values (Kuttappan et al., 2013), in which the pathogenicity of *Leucocytozoon* depended on the levels of infection (Smith et al., 2015). The potential effects of many mechanism on individuals infected with haemosporidian parasites are anemia including parasite-synthesized factors that increase hemolysis and phagocytosis. In addition, the PCV has been used as a variable to evaluate overall avian condition, but most studies have been descriptive without any definitive conclusion (Astudillo et al., 2013). The non-significant differences among the three phylogenetic groups of leucocytozoid infection might have been due to the low numbers of *Leucocytozoon*-infected chickens in groups II and III.

The morphology of leucocytozoids of chickens and FCs in the current study (Groups I–III) had the main diagnostic characters of the morphospecies *L. sabrazesi* or *L. macleani* which develop both elongate and roundish host cells (Valkiūnas, 2005). This appearance may be associated with the vectors of *L. sabrazesi* in Thailand, namely *Cullicoides* spp. and *Simulium* spp. (Valkiūnas, 2005; Tongkamsai and Napoon, 2015). The phylogenetics of the PK and KPS leucocytozoids were not attributed to *L. sabrazesi* and *L. caulleryi* that have been reported in this area (Worasing et al., 2001; Tongkamsai and Napoon, 2015) because the cyt *b* genetic divergences among lineages in the current study from *L. sabrazesi* and *L. caulleryi*, were large (8.9–20.4%). These were similar to the high sequence divergences (1.5%) of *L. schoutedeni* in Uganda and Cameroon (Sehgal et al., 2006b) where there were no morphological differences between blood stages of the parasites represented by the two different lineages. Two distinct clades of *L. danilewskyi* in boreal owls (*Aegolius funereus*) from Lithuania showed identical morphologies (Ishak et al., 2008). Sehgal et al. (2006a) also reported a high sequence divergence (10.9%) between two lineage clades of *L. toddi* in *Buteo* spp. and *Accipiter* spp. from California, USA. This combined evidence supported the cryptic species of *Leucocytozoon* spp. (Ishak et al., 2008). The phylogeny and ecology suggested that the leucocytozoids in the current study were cryptic species or might not be the species *L. sabrazesi*.

The leucozytozoils isolated from the PK and KPS chickens had a close genetic lineage to the parasites found in chicken from Malaysia and a sister group of *L. schoutedeni* strains in Malaysia (Valkiūnas, 2005), Uganda and Cameroon (Sehgal et al., 2006b). This observation raises the issue of whether these incidents involving the same species of *Leucocytozoon* in chicken from Malaysia was not the same species of *L. schoutedeni* that developed only roundish host cells (Valkiūnas, 2005; Sehgal et al., 2006b). One report attempted to elucidate the relationship of environmental change, occupancy and genetic diversity to indicate the host specialists and generalists that were successful in colonizing in all study regions (Drovetski et al., 2014).

The sequences of *Leucocytozoon* spp. were scant; only the cyt *b* gene has enough data to compare species and genetic distribution. Martinsen et al. (2008) advised that a future study should include the four leading genes to form a strong node. The morphological markers available for differential diagnosis for *Leucocytozoon* are not clear-cut and their interpretation might become ambiguous (Paperna et al., 2008). The sensitive PCR described remarkably more parasite diversity than previously expected, similar to those reported of *Haemoproteus* spp. (Valkiūnas et al., 2014).

In conclusion, microscopy and molecular study revealed the similar morphology and phylogenetic groups of *Leucocytozoon* in both chickens and FCs. The hematology revealed the low pathogenicity of the leucocytozoids in chickens and FCs. The genetic divergences among the sequences in the current study and *L. sabrazesi* were large, so they might be cryptic species or not be the same species. This study should help to define the intraspecific diversity and phylogenetic relationships of chicken leucocytoziods in the future.

Conflict of Interest

The authors declare that there are no conflicts of interest.

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