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Molecular characterization of *Plasmodium juxtanucleare* in Burmese red junglefowls (*Gallus gallus spadiceus*) in Thailand

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ABSTRACT

Plasmodium gallinaceum and *Plasmodium juxtanucleare* are the most common species of *Plasmodium* causing avian malaria in domestic and wild chickens. In Thailand, *P. gallinaceum* infection in poultry has been reported since 1996, but *P. juxtanucleare* infection has never been reported. The objective of this study was to identify and characterize the *Plasmodium* species infecting Burmese red junglefowls (*Gallus gallus spadiceus*) in Thailand using molecular techniques. Seventeen Burmese red junglefowls from a farm in western Thailand were suspected of being infected with blood parasite. Blood samples of all seventeen Burmese red junglefowls were collected and examined. Microscopic examination revealed the presence of *Plasmodium* trophozoites in 5 out of 17 samples. In comparison, nested PCR detected 12 samples to be positive for *Plasmodium* spp. Molecular characterization, based on partial sequence of mitochondrial cytochrome *b* gene, revealed 7 PCR-positive samples as *P. juxtanucleare*. Nucleotide sequence comparison and phylogenetic analyses grouped these 7 *P. juxtanucleare* into 2 distinct groups. The first group was identical to *P. juxtanucleare* previously reported from Brazil, Malaysia and Taiwan, whereas the second group was identical to *P. juxtanucleare* from Japan. This is the first report on molecular confirmation and characterization of *P. juxtanucleare* in Burmese red junglefowls in Thailand.

Keywords: Avian malaria, Burmese red junglefowl, cytochrome *b* gene, *Plasmodium juxtanucleare*, Thailand

INTRODUCTION

Avian malaria is a mosquito-transmitted disease of domestic and wild birds found worldwide. The disease is caused by more than 40 species of the blood parasites in the genus *Plasmodium* that differ broadly across hosts, vectors, geography, and pathogenicity. Infected birds are typically anemic, lethargic, anorexic, and have ruffled feathers. Without treatment, the disease can lead to death (Atkinson, 2008). *P. gallinaceum* and *P. juxtanucleare* are the most common species causing avian malaria in domestic chickens and wild fowls, especially the red junglefowls (*Gallus gallus*) (Taylor *et al*., 2007; Atkinson, 2008). *P. gallinaceum* is pre-dominantly found in the Southeast Asia, whereas *P. juxtanucleare* is found in regions such as the Central and South America, East and South Africa, and Asia (Taylor *et al*., 2007). In Thailand, the first outbreak of avian malaria caused by *P. gallinaceum* was reported in 1996 (Chompoochan *et al*., 1996; Mahantachaisakul *et al*., 1996). However, avian malaria caused by *P. juxtanucleare* has never been reported in Thailand, even though the infections of *P. juxtanucleare* have been reported in neighboring countries such as Malaysia (Fernando and Dissanaike, 1975) and Vietnam (Kissinger *et al*., 2002). *P. juxtanucleare* was first observed in blood samples from domestic chickens (*Gallus gallus domesticus*) in Brazil in

1941 (Versiani and Gomes, 1941). Besides domestic chickens, there have been several reports of other fowls being naturally infected by *P. juxtanucleare* such as red junglefowl in Malaysia (Fernando and Dissanaike, 1975), Bamboo Partridge in Taiwan (Manwell *et al*., 1976), greywing francolin and black-footed penguin in South Africa (Earle *et al*., 1991; Grim *et al*., 2003), and white eared-pheasant in Japan (Murata *et al*., 2008). Red junglefowl, a member of the Phasianidae family, is a native species in the South and Southeast Asia. Its two subspecies, including Indochina red junglefowl (*G. g. gallus*) and Burmese red junglefowl (*G. g. spadiceus*), are found in Thailand and are considered an endangered species (Akaboot *et al*., 2012).

The gold standard for diagnosis of *Plasmodium* infection is microscopy by Giemsa-stained thin blood smear. Microcopy can reveal the presence of trophozoites, merozoites and gametocytes of the *Plasmodium* parasites in erythrocytes (Valkiunus, 2004; Atkinson, 2008). Several PCR methods for detecting avian malaria parasites in blood samples have been described to date. Most are based on amplification of conserved fragments of either the 18S small-subunit ribosomal RNA gene (*18S rRNA*) (Feldman *et al*., 1995; Jarvi *et al*., 2002) or mitochondrial cytochrome *b* gene (*cyt b*) (Hellgren *et al*., 2004; Waldenstrom *et al*., 2004; Valkiunas *et al*., 2009). Partial and whole *cyt b* gene sequences of various *Plasmodium* species are available in NCBI GenBank (www.ncbi.nlm.nih.gov/genbank/) and MalAvi (http://mbio-serv2.mbioekol.lu.se/Malavi/) databases, which can be used for species identification by nucleotide sequence comparison (Bensch *et al*., 2009). The purpose of this study was to identify and characterize the *Plasmodium* species infecting Burmese red junglefowls in Thailand using molecular techniques based on partial sequence of *cyt b* gene.

MATERIALS AND METHODS

Blood sample collection

In November 2014, poultry keepers at a farm in western Thailand noticed that 10 domestic chickens raised around Burmese red junglefowls' pens had diarrhea with white droppings followed by convulsions and death within a couple of days. A few days later, an additional 17 Burmese red junglefowls became lethargic. Blood samples of these 17 Burmese red junglefowls were collected and submitted to the Parasitology Section at the National Institute of Animal Health (NIAH) for diagnosis (EA-007/59(R)).

Preparation of stained thin smear and microscopic examination

Thin blood films were prepared from each blood sample, fixed in absolute methanol for 5 min, allowed to dry, and subsequently stained with 10% Giemsa's stain for 30 min. The stained smear slides were then washed under tap water and air-dried. Each slide was examined under a Nikon eclipse Ni light microscope (Nikon Corp., Tokyo, Japan) at a magnification of 1,000× with oil immersion. Avian malaria parasites were identified according to Valkiunas (2004). Microphotographs of the blood parasites were taken using a Nikon D5-Fi2 digital camera mounted on the light microscope (Nikon Corp.).

DNA extraction and nested polymerase chain reaction (nested PCR)

DNA samples were extracted from all 17 blood samples using Genomic DNA Mini Kit (Blood/Cultured cell) (Geneaid Biotech Ltd., New Taipei City, Taiwan) following the manufacturer's instruction. The extracted DNA samples were used as templates in the nested PCR method described by Hellgren *et al*. (2004) for detection of the *cyt b* gene of *Plasmodium* spp. In the first-round PCR, a part of the *cyt b* gene with 618 bp in length, corresponding to nucleotide positions 186 to 803 of *P. juxtanucleare cyt b* gene (AB250415), was amplified using outer primers Haem NFI (5′-CAT ATA TTA AGA GAA ITA TGG AG-

3′) and Haem NR3 (5′-ATA GAA AGA TAA GAA ATA CCA TTC-3′). The PCR reaction was carried out in a Veriti® 96-Well Thermal Cycler (Applied Biosystems, Foster City, CA, USA) using a GoTaq® Green Master Mix kit (Promega, Madison, WI, USA) according to the manufacturer's instruction. Briefly, 2 μl of extracted DNA sample was added as template to 23 μl of a PCR reaction mixture, which consisted of 12.5 μl of GoTaq® Green Master Mix solution, 0.5 μl each of 10 μM forward and reverse primers, and 9.5 μl of nuclease free water. The thermal cycling condition comprised one cycle of initial denaturation at 95°C for 2 min, followed by 20 cycles of amplification at 95°C for 30 sec, 49°C for 30 sec and 72°C for 45 sec, and one cycle of final extension at 72°C for 10 min. In the second-round PCR, inner primers Haem F (5′-ATG GTG CTT TCG ATA TAT GCA TG-3′) and Haem R2 (5′-GCA TTA TCT GGA TGT GAT AAT GGT-3′) were used. The composition of the PCR reaction mixture was the same as that of the first-round PCR. The thermal cycling conditions were the same as those described for the first-round PCR except the number of cycles was increased to 35 cycles. The PCR products were analyzed by gel electrophoresis on a 1.5% agarose gel. The gel was then stained with 0.5 mg/ml ethidium bromide for 30 min and washed with water before visualizing the bands of PCR products under an UV illuminator. The sample containing a band of PCR product with an expected size of 525 bp was considered positive for *Plasmodium* spp.

Nucleotide sequencing

The first-round PCR product of the PCR-positive samples were re-amplified. The PCR products were purified using FavorPrep™ GEL/PCR purification kit (Favorgen biotech corp., Ping-Tung, Taiwan) according to the manufacturer's instructions. The purified PCR products were sequenced directly using the outer primer set with the Big Dye termination cycle kit version 3.1® (Applied Biosystems) in an ABI Prism 3130 Genetic Analyzer (Applied Biosystems). Each purified PCR product was sequenced from both directions giving forward and reverse sequence reads. The obtained nucleotide sequences were assembled using BioEdit software version 7.1.11 (Hall, 1999). The assembled sequences were deposited in GenBank database using BankIt tool (www.ncbi.nlm.nih.gov/WebSub/?tool=genbank).

Analysis of nucleotide sequence data

The assembled *cyt b* gene sequences (excluding the outer primer binding sites) were identified by comparing with nucleotide sequences in NCBI GenBank database using basic local alignment tool (BLASTN) (https://blast.ncbi.nlm.nih.gov/Blast.cgi). The identified sequences were then analyzed by comparing with sequences of the matching *Plasmodium* species along with other *Plasmodium* species obtained from the GenBank database listed in Table 1. Percent identity and multiple sequence alignment analyses of the nucleotide and deduced amino acid sequences were generated using BioEdit software. The phylogenetic tree of nucleotide sequences was constructed using MEGA6 software version 6.06 (Tamura *et al*., 2013) by the maximum likelihood method based on Tamura 3-parameter (Tamura *et al*., 1992) with 1,000 replications of bootstrap values and rooted with *cyt b* gene sequence of *Leucocytozoon sabrazesi* (AB299369). Multiple sequence alignment analysis was performed based on the 571 bp region corresponding to nucleotide positions 209 to 779. Phylogenetic and percent identity analyses were performed using an alignment of the 450 bp region corresponding to positions 260 to 709 that all of the samples had in common except a sample from Vietnam (EF011196), which was 342 bp in length.

Table 1. Description of *Plasmodium* spp. included in this study.

***Plasmodium* species**	**Host species**	**Geographic origin**	**GenBank accession no.**
P. juxtanucleare -TH41	*G. g. spadiceus*	Thailand	KU248841
P. juxtanucleare -TH44	*G. g. spadiceus*	Thailand	KU248842
P. juxtanucleare -TH47	*G. g. spadiceus*	Thailand	KU248843
P. juxtanucleare -TH50	*G. g. spadiceus*	Thailand	KU248844
P. juxtanucleare -TH51	*G. g. spadiceus*	Thailand	KU248845
P. juxtanucleare -TH52	*G. g. spadiceus*	Thailand	KU248846
P. juxtanucleare -TH58	*G. g. spadiceus*	Thailand	KU248847
P. juxtanucleare	*Gallus gallus*	Brazil	DQ017964
P. juxtanucleare	*Gallus gallus*	Brazil	KC142195
P. juxtanucleare	n.a.	Japan	AB250415
P. juxtanucleare	*Crossoptilon crossoptilon*	Japan	AB302893
P. juxtanucleare	*G. g. spadiceus* / *G. g. domesticus*	Malaysia	KT290907
P. juxtanucleare	*G. g. spadiceus* / *G. g. domesticus*	Malaysia	KT290908
P. juxtanucleare	*G. g. spadiceus*	Malaysia	KT290909
P. juxtanucleare	*G. g. spadiceus*	Malaysia	KT290910
P. juxtanucleare	*G. g. spadiceus*	Malaysia	KT290911
P. juxtanucleare	*G. g. spadiceus*	Malaysia	KT290912
P. juxtanucleare	*G. g. spadiceus*	Malaysia	KT290913
P. juxtanucleare	*G. g. spadiceus*	Malaysia	KT290914
P. juxtanucleare	*G. g. spadiceus*	Malaysia	KT290915
P. juxtanucleare	*G. g. spadiceus*	Malaysia	KT290916
P. juxtanucleare	*G. g. spadiceus*	Malaysia	KT290917
P. juxtanucleare	*G. g. spadiceus*	Malaysia	KT290918
P. juxtanucleare	*G. g. spadiceus*	Malaysia	KT290919
P. juxtanucleare	*Gallus gallus*	Malaysia	KT290920
P. juxtanucleare	*Gallus gallus*	Malaysia	KT290921
P. juxtanucleare	n.a.	Philippines	KP689332
P. juxtanucleare	n.a.	Taiwan	KP326567
P. juxtanucleare	*Gallus gallus*	Vietnam	EF011196
P. gallinaceum	n.a.	Thailand	KP025675
P. circumflexum	*Sylvia atricapilla*	n.a.	JN164734
P. relictum	*Hemignathus virens*	USA	AY733090
P. cathemerium	*Serinus canaria*	Germany	AY377128
P. elongatum	*Passer domesticus*	North America	AF069611

n.a. = not available

RESULTS AND DISCUSSION

Microscopic examination of the Giemsa-stained thin smears revealed that 5 out of 17 blood samples were positive for blood parasites. However, only trophozoite forms of the parasites were observed in the cytoplasm of the red blood cells. The trophozoites were oval and roundish in shape, contained pigment granules, and were juxtaposed to the nuclei of the red blood cells (Fig. 1). From morphological characteristics, the parasites were identified as *Plasmodium* spp. Infection of *P. gallinaceum* can be identified by the presence of trophozoites, meronts with numerous merozites and round gametocytes that displace the nucleus of the host red blood cell. In comparison, infection of *P. juxtanucleare* can be distinguished from infection of *P. gallinaceum* by the presence of more elongated gametocytes that are smaller than the nucleus of the host red blood cell which do not displace the nucleus of the red blood cell. In addition, the trophozoite, meront and gametocyte of *P. juxtanucleare* have a tendency to adhere closely to the nucleus of the red blood cell (Valkiunas, 2004; Taylor *et al*., 2007). However, the microscopic identification and ability to differentiate the *Plasmodium* spp. requires extensive skill and experience, especially at early and chronic stages of the infection. Thus, it can be difficult to distinguish *P. gallinaceum* from *P. juxtanucleare* because the number of meronts and gametocytes are low at these stages (Silveira *et al*., 2009; 2013). In the present study, the Burmese red junglefowls have been lethargic for only a few days before the blood samples were collected, indicating that the infection of *P. juxtanucleare* in these junglefowls was at the early stage of infection. Therefore, trophozoites of *P. juxtanucleare* were microscopically found (Fig. 1). This finding is consistent with studies on chickens experimentally infected with *P. juxtanucleare*, which demonstrated that trophozoites were mostly observed at the early stage of infection (Silveira *et al*., 2009; 2013).

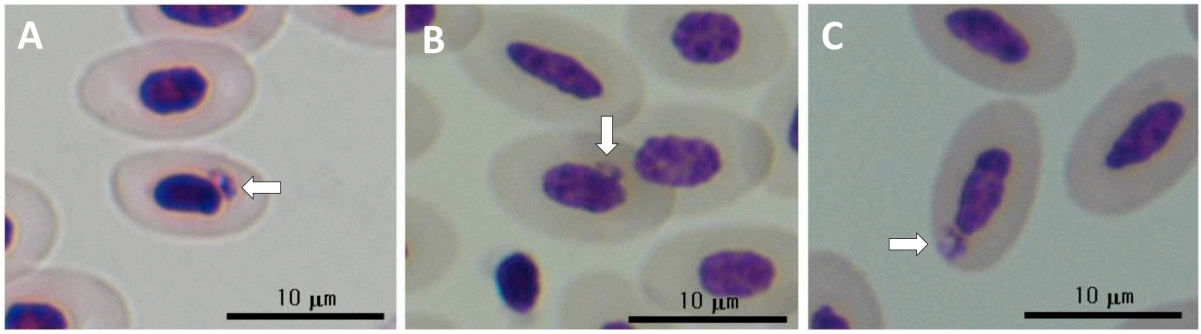

Fig. 1. Giemsa-stained thin smears of Burmese red junglefowl blood samples (×1,000). Oval (A and B) and round (C) trophozoites are juxtaposed to the nuclei of red blood cells, indicating by white arrows.

The microscopic finding of *Plasmodium* spp. in red blood cells of the Burmese red junglefowls was confirmed by nested PCR results. A total of 12 out of 17 samples, including all positive samples from the microscopic examination, were positive for the *cyt b* gene from *Plasmodium* spp., which is consistent with a previous study described by Hellgren *et al*. (2004). The number of PCR-positive samples was higher than the number of positive samples from microscopic examination, indicating that the sensitivity of the nested PCR detection was higher than that of microscopic examination. This result was similar to the study reported by Krams *et al*. (2012), which found that the prevalence of *Plasmodium* spp. in Siberian Tit (*Poecile cinctus*) determined by nested PCR was significantly higher than the prevalence determined by microscopic examination. Therefore, it is recommended that nested PCR should be included in the diagnostic procedures for detection of *Plasmodium* infection in suspected cases.

The 618-bp PCR product from all positive samples were sequenced. The sequences of 7 samples showed clear sequencing chromatograms. These sequence data were then deposited in NCBI GenBank database under accession numbers KU248841-KU248847 (Table 1). However, nucleotide sequences of the other 5 PCR-positive samples showed ambiguous sequencing chromatograms, which suggested mixed infection; therefore, these 5 samples were discarded from the study. Even though, the blood samples in this study were taken from only one geographical location, nucleotide sequence comparison analysis among the 7 samples revealed that 2 distinct groups of *P. juxtanucleare* existed in the Burmese red junglefowls in western Thailand. The first group contained 4 samples, designated as TH41, TH47, TH52, and TH58. The second group contained the other 3 samples, TH44, TH50, and TH51. BLASTN analysis of the 7 sequences resulted in 98 – 100% identity with those of *P. juxtanucleare* in NCBI GenBank database. As a result, these *Plasmodium* parasites were identified as *P. juxtanucleare*.

The percent identity between the partial *cyt b* gene sequences of the first and second groups was 99.8%, whereas the overall percent identity among the partial *cyt b* gene sequences of all *P. juxtanucleare* isolates was from 98.2 to 100%. Similarly, the percent identity between the deduced amino acid sequences of the first and second groups was 100%, whereas the overall percent identity among the deduced amino acid sequences of all *P. juxtanucleare* isolates was from 96.6 to 100% (Data not shown).

The multiple nucleotide sequence alignments of the *Plasmodium* sequences revealed that the *cyt b* gene in the studied region were highly conserved among *P. juxtanucleare* when compared to those regions of other *Plasmodium* species (Data not shown). There was only one nucleotide difference between the Thai *P. juxtanucleare* groups 1 and 2 at position 533 (Table 2), resulting in a synonymous amino acid substitution at position 178. Similarly, two distinct groups were also found in *P. juxtanucleare* from Brazil. The difference between the two Brazilian groups was found at position 665 (Table 2), resulting in a substitution of phenylalanine by leucine at position 222. Greater genetic diversity was observed among the sequences of *P. juxtanucleare* from Malaysia which may be due to a larger number of samples tested. Interestingly, all of these sequences contained cytosine (C) at positions 218 and 222 instead of thymine (T) and adenine (A), respectively (Table 2). This indicates that cytosines at these 2 positions could serve as a molecular marker for identification of *P. juxtanucleare* from Malaysia. Therefore, molecular surveillance with a larger number of samples in Thailand is required in order to identify genetic markers of Thai *P. juxtanucleare*, which will provide useful information for outbreak investigation and control in the future.

The phylogenetic analysis revealed that all *P. juxtanucleare* sequences were clustered in the same clade and separated from other *Plasmodium* species (Fig. 2). This result was similar to the phylogenetic studies conducted by Murata *et al*. (2008), Silveira *et al*. (2013), and Chen *et al*. (2015). In addition, the phylogenetic tree showed that the first group was the most closely related to *P. juxtanucleare* from Brazil (KC142195), Malaysia (KT290910), and Taiwan (KP326567), whereas the second group was the most closely related to *P. juxtanucleare* from Japan (AB250415 and AB302893). It is not clear if the two groups of *P. juxtanucleare* in the western Thailand is a result of natural mutation or two different groups were introduced into this area.

In conclusion, *P. gallinaceum* was the only *Plasmodium* species that has been reported to be the causative pathogen of avian malaria in Thailand. Along with the microscopic examination, this study presents the first molecular confirmation and characterization of *P. juxtanucleare* found in Burmese red junglefowls in Thailand. The importance of this finding relates to the possibility that *P. juxtanucleare* might have been infecting chickens in Thailand, but it was not microscopically diagnosed or was misdiagnosed as *P. gallinaceum*. Currently, nucleotide sequence analysis is the only

molecular technique that can differentiate between *P. gallinaceum* and *P. juxtanucleare*. The development of species identification method, such as restriction fragment length polymorphism assay, will be useful for laboratories that are lacking DNA sequencing facility. Moreover, further studies with a larger number of samples from both avian hosts and vectors in different geographical areas of Thailand should be conducted in order to determine the status and the genetic diversity of avian malaria parasites in Thailand and also to monitor the emergence of the exotic species of *Plasmodium* parasites.

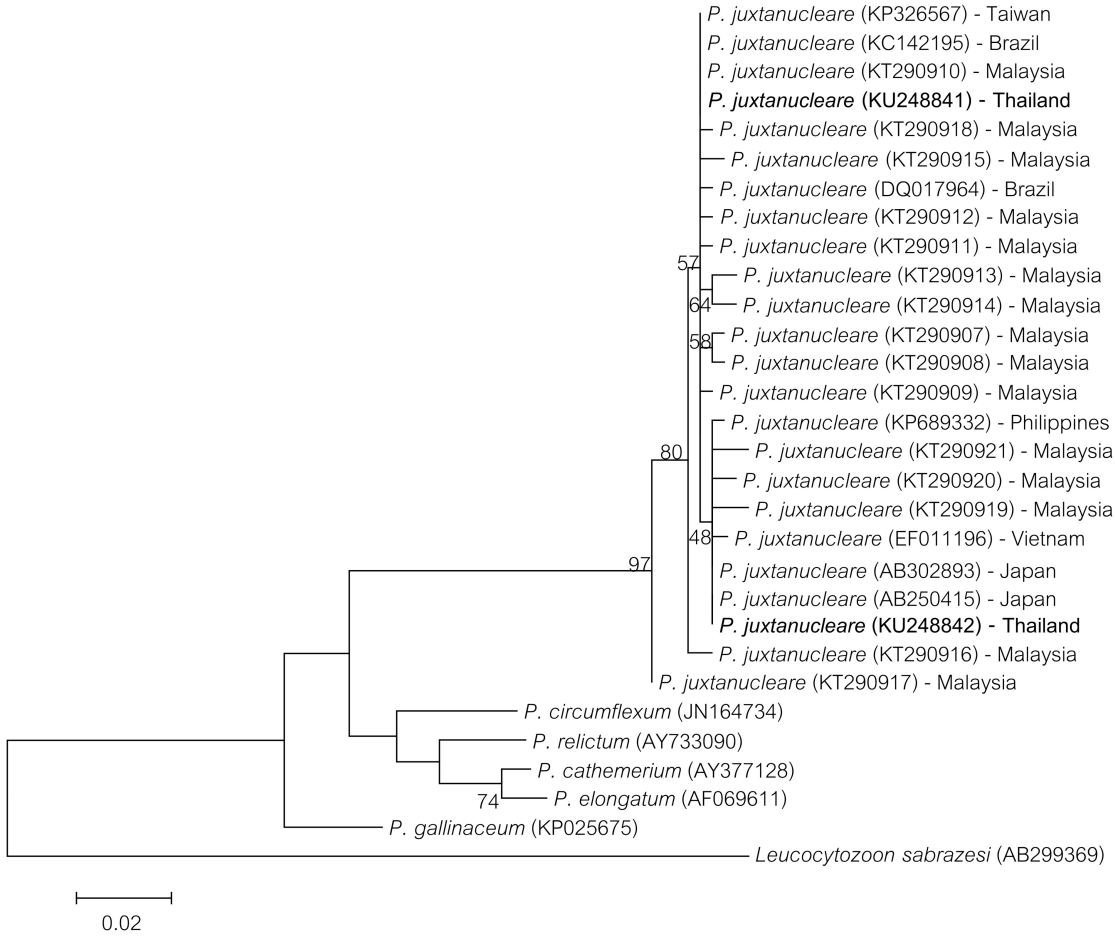

Fig. 2. Phylogenetic tree was constructed by the maximum likelihood method based on partial sequences of *cyt b* gene of *Plasmodium* parasites with 1,000 replications of bootstrap values and rooted with *cyt b* gene sequence of *Leucocytozoon sabrazesi* (GenBank accession no. AB299369). KU248841 and KU248842 are the representatives of the Thai *P. juxtanucleare* groups 1 and 2, respectively.

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Table 2. Multiple nucleotide sequence alignment result showing only variable nucleotide positions between the partial cty *b* gene sequences of *P. juxtanucleare* from Thailand and those of other *P. juxtanucleare* obtained from NCBI GenBank database. Nucleotides that differ from *P. juxtanucleare* accession number KU248841 are indicated by grey shading. Dashes (-) indicate no data. KU248841 and KU248842 are the representatives of the Thai *P. juxtanucleare* groups 1 and 2, respectively.

Accession number	Geographic origin	Nucleotide position																																				
		218	222	245	261	263	266	269	271	275	293	305	324	338	341	416	478	513	515	518	528	533	539	562	573	585	591	604	613	631	638	662	665	692
KU248841	Thailand	T	A	A	C	T	T	T	T	T	T	A	A	A	A	T	T	T	A	T	C	T	T	T	A	G	A	T	T	A	T	A	T	A
KU248842	Thailand	T	A	A	C	T	T	T	T	T	T	A	A	A	A	T	T	T	A	T	C	C	T	T	A	G	A	T	T	A	T	A	T	A
DQ017964	Brazil	-	-	A	C	T	T	T	T	T	T	A	A	A	A	T	T	T	A	T	C	T	T	T	A	G	A	T	T	A	T	A	G	A
KC142195	Brazil	-	-	A	C	T	T	T	T	T	T	A	A	A	A	T	T	T	A	T	C	T	T	T	A	G	A	T	T	A	T	A	T	A
AB250415	Japan	T	A	A	C	T	T	T	T	T	T	A	A	A	A	T	T	T	A	T	C	C	T	T	A	G	A	T	T	A	T	A	T	A
AB302893	Japan	-	-	A	C	T	T	T	T	T	T	A	A	A	A	T	T	T	A	T	C	C	T	T	A	G	A	T	T	A	T	A	T	A
KT290907	Malaysia	C	C	A	C	T	T	T	T	T	T	A	A	A	A	T	T	T	A	C	C	T	T	T	G	G	A	T	T	A	T	A	T	A
KT290908	Malaysia	C	C	A	C	T	T	T	T	T	T	A	A	T	A	T	T	T	A	C	C	T	T	T	A	G	A	T	T	A	T	A	T	A
KT290909	Malaysia	C	C	A	C	T	T	T	T	T	T	A	A	A	A	T	T	T	A	T	C	T	T	T	T	G	A	T	T	A	T	A	T	A
KT290910	Malaysia	C	C	A	C	T	T	T	T	T	T	A	A	A	A	T	T	T	A	T	C	T	T	T	A	G	A	T	T	A	T	A	T	A
KT290911	Malaysia	C	C	A	C	T	C	T	T	T	T	A	A	A	A	T	T	T	A	T	C	T	T	T	A	G	A	T	T	A	T	A	T	A
KT290912	Malaysia	C	C	A	C	T	T	T	T	T	T	A	A	A	A	T	T	T	A	T	C	T	T	T	A	G	A	T	T	A	T	G	T	A
KT290913	Malaysia	C	C	A	C	T	T	T	T	T	C	A	A	A	A	T	G	T	A	T	C	T	T	T	A	G	A	C	T	A	T	A	T	A
KT290914	Malaysia	C	C	A	C	T	T	T	T	T	C	A	A	A	A	T	T	T	G	T	C	T	T	T	A	A	A	T	T	A	T	A	T	A
KT290915	Malaysia	C	C	A	C	T	T	T	T	T	T	A	A	A	A	T	T	C	A	T	C	T	T	T	A	G	A	T	T	A	C	A	T	A
KT290916	Malaysia	C	C	A	C	T	T	C	C	T	T	A	G	A	A	T	T	T	A	T	C	T	T	T	A	G	A	T	T	A	T	A	T	A
KT290917	Malaysia	C	C	T	T	A	T	C	T	T	T	A	A	A	A	T	T	T	A	T	C	T	T	C	A	G	A	T	T	A	T	A	T	A
KT290918	Malaysia	C	C	A	C	T	T	T	T	T	T	A	A	A	A	T	T	T	A	T	C	T	T	T	A	G	A	T	T	A	T	A	T	G
KT290919	Malaysia	C	C	A	C	T	T	T	T	T	T	A	A	A	G	T	T	T	A	T	T	C	T	T	A	G	A	T	T	G	T	A	T	A
KT290920	Malaysia	C	C	A	C	T	T	T	T	T	T	A	A	A	A	T	T	T	A	T	C	C	C	T	A	G	A	T	C	A	T	A	T	A
KT290921	Malaysia	C	C	A	C	T	T	T	T	C	T	G	A	A	A	T	T	T	A	T	C	C	T	T	A	G	G	T	T	A	T	A	T	A
KP689332	Philippines	-	-	-	C	T	T	T	T	T	T	A	A	G	A	T	T	T	A	T	C	C	T	T	A	G	A	T	T	A	T	A	T	A
KP326567	Taiwan	-	-	-	C	T	T	T	T	T	T	A	A	A	A	T	T	T	A	T	C	T	T	T	A	G	A	T	T	A	T	A	T	A
EF011196	Vietnam	T	A	A	C	T	T	T	T	T	T	A	A	A	A	A	T	T	A	T	C	C	T	T	A	G	A	-	-	-	-	-	-	-

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

SUBMISSION DECLARATION AND VERIFICATION

The authors declare that this manuscript is original, has not been published before and is not currently being considered for publication elsewhere.

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