

Molecular characterization of *Plasmodium juxtanucleare* in Thai native fowls based on partial cytochrome C oxidase subunit I gene

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Abstract: Avian malaria is one of the most important general blood parasites of poultry in Southeast Asia. *Plasmodium* (*P.*) *juxtanucleare* causes avian malaria in wild and domestic fowl. This study aimed to identify and characterize the *Plasmodium* species infecting in Thai native fowl. Blood samples were collected for microscopic examination, followed by detection of the *Plasmodium* cox I gene by using PCR. Five of the 10 sampled fowl had the desired 588 base pair amplicons. Sequence analysis of the five amplicons indicated that the nucleotide and amino acid sequences were homologous to each other and were closely related (100% identity) to a *P. juxtanucleare* strain isolated in Japan (AB250415). Furthermore, the phylogenetic tree of the cox I gene showed that the *P. juxtanucleare* in this study were grouped together and clustered with the Japan strain. The presence of *P. juxtanucleare* described in this study is the first report of *P. juxtanucleare* in the Thai native fowl of Thailand.

Keywords: fowl, cytochrome C oxidase subunit I, *Plasmodium juxtanucleare*

Introduction

Avian malaria, caused by *Plasmodium* spp., is an important blood parasite disease of poultry because it results in poor meat quality and egg production [1]. The clinical signs of avian malaria in host birds vary from no apparent signs to severe anemia and death, and the mortality rate associated with avian malaria has been reported to be as high as 80%–90% [2]. *Plasmodium* spp. have been reported to infect in various domestic fowl; *Plasmodium* (*P.*) *gallinaceum* occurs in jungle fowl and domestic fowl, while *P. juxtanucleare* has been detected in domestic fowl and turkeys. Avian malaria is common in tropical countries such as Asia, East and South Africa, and Central and South America [3]. *P. juxtanucleare* was first described in Brazil [4] and has since been reported in Asian countries such as Malaysia [5], Vietnam [6], and Thailand [7].

Generally, detection of *Plasmodium* in infected birds can be performed by Giemsa staining of a thin blood smear, which may reveal erythrocytes with trophozoites, merozoites, and/or gametocytes of *Plasmodium* spp. [8]. That method is performed by specialist workers familiar with detecting and identifying parasites in blood samples. In addition, a DNA-based detection method for identifying avian malaria parasites in blood samples has been reported [9]. The mitochondrial genome of apicomplexan parasite encodes three protein-coding genes (cytochrome b [cyt b], cytochrome c oxidase subunits I [cox I] and III [cox III]) and six fragments of large subunit rRNA genes. The cyt b and cox I genes have been widely used in the molecular detection of avian malaria parasites such as *P. gallinaceum* [10,11]. The cox I gene is the target region used for species identification and phylogenetic analysis because it is the most highly conserved mitochondrially encoded protein [12–14]. In Thailand, a molecular characterization study of *P. juxtanucleare* reported only a partial sequence of the cyt b gene of Burmese red jungle fowls [7]. To date, there are no cox I gene-based reports on *P. juxtanucleare* infection in Thai native fowl. Therefore, the aim of this study was to identify and characterize the malaria parasites infecting Thai native fowl by using molecular tech-

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Conflict of Interest

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niques based on the partial cox I gene.

Materials and Methods

Blood sample collection

In 2016, ten of 50 Thai native fowl that had been raised in a backyard in northeast Thailand exhibited paleness and emaciation. These 10 fowl were suspected to be exhibiting sub-clinical signs of a blood parasite infection. Thus, blood samples were collected and submitted to the Veterinary Diagnostic Laboratory, Faculty of Veterinary Medicine, Khon Kaen University, Thailand.

Blood smear analysis

The collected blood smears were prepared as thin blood films and fixed with absolute methanol for 10 min, left to dry, and then stained with 10% Giemsa solution for 30 min. The stained slides were then washed with tap water and air-dried. Each slide was examined under a light microscope (Olympus, Tokyo, Japan) using 1,000× magnification with oil immersion. Avian malaria parasites were observed and identified as described by Valkiunas [11].

DNA extraction and polymerase chain reaction (PCR)

The DNA of each of the 10 malaria-suspected fowl was extracted from each blood sample by using a TIANamp Blood DNA Maxi Kit (TIANGEN Biotech, Beijing, China) following the manufacturer's instruction. The PCR method described by Hellgren et al. [15] for detection of the cox I gene of *Plasmodium* spp. was used. A partial cox I gene with a length of 588 base pairs (bp) was amplified by using forward primer CoxI-F1 (5'-GCGTACTTTGGACCGAAAA-

3') and reverse primer CoxI-R1 (5'-CATCCAGTACCAC-CACCAAA -3') as described in a previous study of cox I genes of *P. gallinaceum* in Thailand [9]. The PCR was performed in a DNA thermocycler (Major cycler, Major Science, Taiwan) using a DreamTaq Green PCR Master Mix (2×) (ThermoFisher Scientific, USA) following the manufacturer's instruction. Briefly, 3 µL of each DNA sample was added to 22 µL of the PCR mixture, which was composed of 12.5 µL of DreamTaq Green PCR Master Mix solution, 0.5 µL of 10 µM of each forward and reverse primer, and 8.5 µL of nuclease-free water. Thermal cycling was carried out under the optimized conditions of 95°C for 5 min, followed by 40 cycles of 95°C for 30 sec, 55°C for 30 sec, and 68°C for 60 sec, followed by a final period at 68°C for 10 min. The PCR products were analyzed by electrophoresis using 1.2% agarose gels containing 1× of GelRed™ Nucleic Acid Gel Stain. Each gel was electrophoresed at 100 volts for 30 min and then visualized by using ultraviolet light in the Gel Doc™ XR+ Gel Documentation System (BioRad, USA).

Nucleotide sequence analysis of the cox I gene

The PCR products were purified for sequencing by using a NucleoSpin Gel and PCR Clean-up (Macherey-Nagel, Germany) kit following the manufacturer's instructions. Sequencing was performed by First BASE Laboratories Sdn Bhd, Malaysia). The obtained nucleotide sequences were analyzed to determine sequence identities by using the BLASTN program (<https://www.ncbi.nlm.nih.gov/BLAST>) and were assembled by using BioEdit software version 7.1.11 [16]. The BankIt tool was used for analysis of the sequence data of the 588 bp fragment submissions in the GenBank database (<https://www.ncbi.nlm.nih.gov/WebSub/?tool=genbank>). The

Table 1. GenBank accession numbers of reference strains of *Plasmodium* species and *Leucocytozoon* species, country of origin used in neighbor-joining analysis of cox I sequences

<i>Plasmodium</i> species	Host species	Geographic origin	GenBank accession number
<i>P. juxtamucleare</i> -KKUTH02	Chicken	Thailand	KY930301
<i>P. juxtamucleare</i> -KKUTH03	Chicken	Thailand	KY930302
<i>P. juxtamucleare</i> -KKUTH04	Chicken	Thailand	KY930303
<i>P. juxtamucleare</i> -KKUTH07	Chicken	Thailand	KY930304
<i>P. juxtamucleare</i> -KKUTH10	Chicken	Thailand	KY930305
<i>P. juxtamucleare</i>	n.a	Japan	AB250415
<i>P. gallinaceum</i>	n.a	Philippine	AB250690
<i>P. gallinaceum</i>	n.a	Thailand	KP025674
<i>P. gallinaceum</i>	n.a	Japan	AB599930
<i>P. relictum</i>	n.a	United Kingdom	LN835311
<i>P. relictum</i>	Spheniscus demersus	South Africa	KY653774
<i>P. circumflexum</i>	Troglodytes troglodytes	Lithuania	KY653762
<i>P. elongatum</i>	Spheniscus demersus	South Africa	KY653802
<i>Leucocytozoon caulleryi</i>	n.a	Japan	AB302215
<i>Leucocytozoon sabrazesi</i>	n.a	Malaysia	AB299369

P. juxtamucleare, *Plasmodium juxtamucleare*; n.a, not available; *P. gallinaceum*, *Plasmodium gallinaceum*; *P. relictum*, *Plasmodium relictum*; *P. circumflexum*, *Plasmodium circumflexum*; *P. elongatum*, *Paramonostomum elongatum*.

sequences of KKUTH02, KKUTH03, KKUTH04, KKUTH07, and KKUTH10 have been deposited in GenBank databases with the accession numbers KY930901, KY930902, KY930903, KY930904, and KY930905, respectively.

Phylogenetic analysis

The identified partial cox I gene sequences were analyzed by comparison with other *Plasmodium* spp. sequences obtained from the GenBank database. GenBank accession numbers for the mitochondrial sequences (Table 1) were used during the analysis. The nucleotide and deduced amino acid sequence identities and multiple sequence alignment analyses were analyzed by using ClustalW of BioEdit software [17]. Distance analyses of and phylogenetic tree generation from the nucleotide sequences were performed by applying the neighbor-joining and maximum likelihood methods by using MEGA6 software version 6.06 [18] based on the Kimura 2-parameter model with 1,000 replications of bootstrap values. *Leucocytozoon caulleryi* and *Leucocytozoon sabraezesi* sequences were used as out-of-group members to root the tree.

Results

Microscopic examination

Examination of the Giemsa-stained blood thin smears revealed that none of the ten Thai native fowl sampled had visible plasmodial stages in the blood smears.

Homology analysis

Agarose gel electrophoresis of five of the ten Thai native fowl samples revealed the presence of 588 bp amplicons (Fig. 1). The results of nucleotide sequence and amino acid analyses revealed 100% nucleotide identities of the ampli-

cons to the cox I gene of *P. juxtannucleare*. The sequence alignment of the nucleotide and amino acid sequences (Figs. 2 and 3) of the five amplicons isolated in this study were 100% similar to each other and were closely related to the *P. juxtannucleare* Japan strain (AB250415). The amino acid sequence identity similarities among samples KKUTH02, KKUTH03, KKUTH04, KKUTH07, KKUTH10, and the Japan strain were 100%.

Phylogenetic analysis

The phylogenetic tree of the partial cox I gene of *P. juxtannucleare* based on including 17 sequences of the GenBank

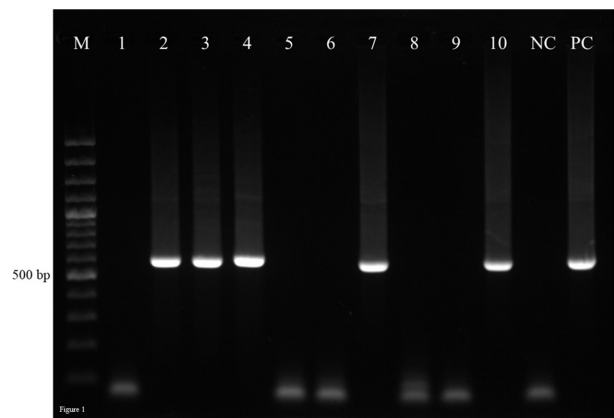


Fig. 1. PCR Electrophoresis gel demonstrating *P. juxtannucleare* cox I gene amplification with CoxI-F1 and CoxI-R1. M. 100 bp DNA marker ladder, Lanes: 1. KKUTH01; 2. KKUTH02; 3. KKUTH03; 4. KKUTH04; 5. KKUTH05; 6. KKUTH06; 7. KKUTH07; 8. KKUTH08; 9. KKUTH09; 10. KKUTH10. NC, negative control; PC, positive control.

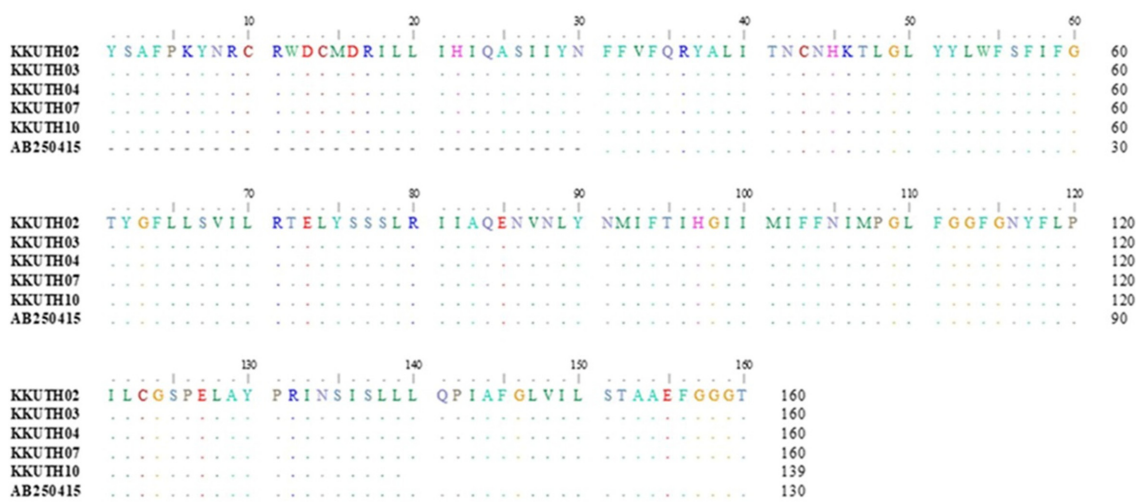


Figure 2

Fig. 2. Deduced amino acid sequences alignment of *P. juxtannucleare* cox I gene from Thai native fowls compared with reference sequence. Dots in the sequence signify the similarity of the amino acids. These sequences of *P. juxtannucleare* Thai native chicken isolates (KKUTH02, KKUTH03, KKUTH04, KKUTH07 and KKUTH10) were identical to that of *P. juxtannucleare* [NCBI reference number: AB250415]. Numbers indicate the amino acid positions.



Figure 3

Fig. 3. Nucleotide sequence of Thai isolates of *P. juxtanucleare* cox I gene. Dots in the sequence signify the similarity of the nucleotides. Nucleotide sequences of *P. juxtanucleare* Thai isolates (KKUTH02, KKUTH03, KKUTH04, KKUTH07 and KKUTH10) were identical to that of *P. juxtanucleare* [NCBI reference number: AB250415]. Numbers indicate the nucleotide positions.

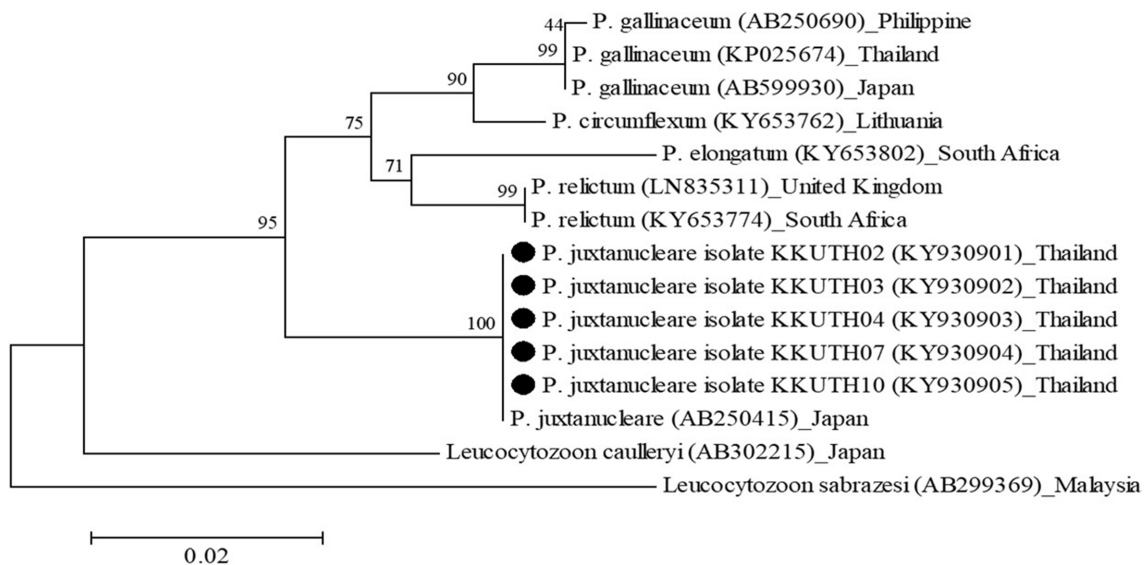


Fig. 4. The phylogenetic tree of the partial cox I gene sequences of *Plasmodium* species. This tree was generated using the neighbor-joining method. Bootstrap values were obtained from 1,000 replications. The scale bar represents the number of substitutions per site. KKUTH02, KKUTH03, KKUTH04, KKUTH07 and KKUTH10 were received from the five positive samples in Thai native folws. Other sequences were acquired from GenBank (Table 1), and *Leucocytozoon caulleryi* and *Leucocytozoon sabrazei* were used as outgroup.

reference strains formed two major groups. *P. juxtanucleare*-positive samples KKUTH02, KKUTH03, KKUTH04, KKUTH07, and KKUTH10 were closely related (100% similarity), and they also had 100% similarity with the *P. juxtanucleare* Japan strain. The five sequences obtained in this study were separated from other *Plasmodium* spp. and from the *Leucocytozoon* species outlier (Fig. 4). They had 95.7%,

95.7%, and 95.5% similarities with *P. gallinaceum* strains from Thailand, Japan, and Philippines, respectively. The greatest phylogenetic distance between our sequences and other published strains were for the partial cox I genes of *P. elongatum*, *P. circumflexum*, and *P. relictum* (95.1%, 95.7%, and 96.1% similarities, respectively) from South Africa, Lithuania, United Kingdom, and USA. The results showed that *P.*

juxtannucleare in Thailand are genetically related to *P. juxtannucleare* of Asian strain.

Discussion

The aim of this study was to identify the presence of *P. juxtannucleare* in Thai native fowls and to compare the partial cox I sequences obtained from our samples with those of other *Plasmodium* spp. isolated around the world. Included in our analyses were various GenBank sequences including one each from wren (*Troglodytes troglodytes*) and black-foot penguin (*Spheniscus demersus*) [19] and that for *P. gallinaceum* isolated from *Gallus gallus domesticus* in Japan [20]. Also included were the PCR amplicons and the percentage identity from the BLASTN software of the five positive field samples. The phylogenetic tree developed for the cox I gene revealed that the five isolates were grouped together and were most similar to the Japan strain of *P. juxtannucleare*. Similarly, the nucleotide and amino acid identities of the five samples (KKUTH02, KKUTH03, KKUTH04, KKUTH07 and KKUTH10) showed a close relationship with the *P. juxtannucleare* isolated in Japan (100% similarity).

Avian malaria is well recognized in poultry, but only a few studies on its molecular characterization have been reported. *P. juxtannucleare* is one of the most common species of *Plasmodium* to cause malaria in poultry. *P. juxtannucleare* has mainly been detected in quail, turkey, and domestic fowls [11,21] in several countries. Several *Plasmodium* spp. cannot be identified based solely on blood stage morphology via microscopic examination, thus molecular technology has to be used for accurate species identification. Moreover, microscopy-based detection of avian malaria presence is less sensitive than the molecular-based method and such sensitivity can be further reduced by low quality blood sample slides or by the inexperience of the investigator. As shown in the present study, PCR assay amplification of the cox I gene fragment can detect *P. juxtannucleare* from fowl blood samples even though the parasite was not detected by light microscopy of blood smears. Therefore, the sensitivity of the PCR assay is higher than that of direct microscopic examination. This finding suggests that PCR assays would be a suitable method for screening fowl for the presence of *P. juxtannucleare* infection.

Plasmodium genes were detected in blood samples by using PCR. A specific PCR fragment of the predicted size (588 bp) was obtained. The partial cox I genes of *Plasmodium* from the sampled Thai native fowl were sequenced and neighbor-joining analysis placed the partial cox I genes into a cluster with a *P. juxtannucleare* isolated from Japan [20]. The finding of *P. juxtannucleare* infection in this study was similar to that in a previous study in Thailand [7]. However, those authors reported molecular characterization of the cyt b gene in infected Burmese red jungle fowls (*Gallus gallus spadiceus*), whereas, our work characterized them based on the cox I gene in Thai native fowl.

A limitation of this study is the small amount of data available in the DNA database for use in comparing strains of the parasite; regardless, our molecular analysis indicated that the parasite isolated is an Asian *Plasmodium* strain. More molecular data of *P. juxtannucleare* and its natural reservoir are required to further describe the evolution and distribution of *Plasmodium*. Herein, we provide the first nucleotide sequence diversity of the cox I gene of *P. juxtannucleare* from field isolates in Thailand. The partial cox I gene analysis sequencing revealed that the *P. juxtannucleare* detected from native fowl in Thailand were very similar to *P. juxtannucleare* isolated in Japan [20]. This study shows the importance of using molecular assays to screen for parasite presence as the fowl in our study had subclinical signs and would have been misdiagnosed based on the microscopic finding. Currently, nucleotide sequence analysis is the only molecular assay that is useful for differentiating between *P. gallinaceum* and *P. juxtannucleare*. The restriction fragment length polymorphism assay was developed for species identification and could be useful for disease diagnosis, but it lacks a DNA sequencing ability. Future studies should investigate samples from commercial poultry that are reared in an area close to backyard birds. Such study will help determine the presence and genetic diversity of *Plasmodium* parasites in commercial farms of Thailand and will assist in monitoring *Plasmodium* spp. presence.

In conclusion, our results provide important information on the detection and phylogenetic relationships of *P. juxtannucleare* present in Thai native fowl in Thailand. The results showed a close relationship among the five isolates and an isolate from Japan. Additionally, the results indicate that the use of PCR-based blood assays is suitable for diagnosis and screening of the *P. juxtannucleare* status in poultry.

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