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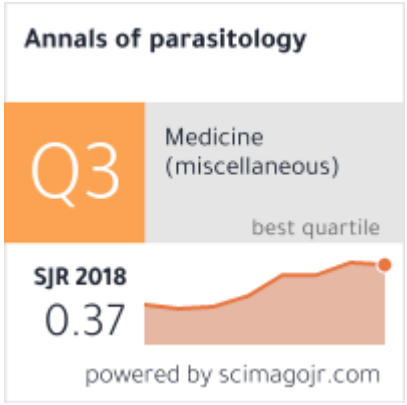

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Original papers

Detection of avian malaria in wild birds at Trisik Beach of Yogyakarta, Java (Indonesia)

Pramana Yuda

Facultas Teknobiologi, Universitas Atma Jaya Yogyakarta, Jl. Babarsari 44, Yogyakarta 55281, Indonesia
E-mail: pramana.yuda@uajy.ac.id

ABSTRACT. Avian haemosporidian (*Plasmodium*, *Haemoproteus* and *Leucocytozoon*) are abundant and widespread vector-borne parasites in birds. However, our knowledge is very limited. This study used a nested-PCR to detect the prevalence level of haematozoan parasites in wild bird at coastal area at Tisik Beach of Yogyakarta, Java Island, Indonesia. In total, 112 DNA samples of 22 species were used. Amplification of cyt-b mtDNA of birds at Trisik beach detected 11 out of 112 samples (9.8 %) of all the blood parasites. Only 5 species out of 22 wild bird species were infected by the avian malaria parasites. Meanwhile, only one out of 20 samples of domestic birds was infected. All positive samples sequenced consistently generated around 450 base pair nucleotides. Alignments of 12 sequences have revealed six parasite lineages in the wild bird at Trisik Beach of Yogyakarta, consist of five lineages for *Plasmodium* sp. and the rest respectively one lineages for *Haemoproteus* sp. and *Leucocytozoon* sp. The results of this study provide additional evidences for *Plasmodium* lineages that uniquely were only infected the Pintail Snipe, Javan Munia, and domestic duck, respectively one lineage, and two lineages in the Yellow Bittern. Meanwhile, *Haemoproteus* and *Leucocytozoon* were not uniquely infecting specific host.

Key words: avian malaria, prevalence, coastal wild birds, nested-PCR, Java Island

Introduction

Avian haemosporidian (*Plasmodium*, *Haemoproteus* and *Leucocytozoon*) are abundant and widespread vector-borne parasites in birds. Over 50 species of these parasites have been identified using light microscopy [1]. Application of molecular methods using PCRs to identify infections has led to improved detection efficiency and has identified over 1300 unique avian haemosporidian lineages [2]. In India/S.E Asia it has been observed 96 and 122 unique lineages respectively for *Plasmodium* and *Haemoproteus*. Furthermore, using the non-parametric Chao2 estimator Clark et al. [3] estimated the lineage diversity in the area was 79 (*Plasmodium*) and 250 (*Haemoproteus*). The report may under-estimate, since the data used for the study from SE Asia is limited from Philippines. Other hotspot areas, i.e. Sundaland (Sumatera, Kalimantan, and Java) and Walacea, were not included in the study.

Java Island of Indonesia is one of the most populated island in the world. However, the island harbours high diversity of wildlife, i.e. 289 bird species [4], and 32 of it is endemic birds [5]. Since 19th century the island has been the subject of considerable ornithological studies [6,7], and accelerated in the last ten years. However, the work on pathogens in the wild bird is very limited. A study on blood parasite has been done to assess the prevalence of avian malaria in forest bird. This study used blood smear method and found that over 50% of the examined bird species were infected by more than one parasite species [8]. Two other studies on estrildid birds applied molecular technique, and detected 25 out of 68 samples (46.7%) for the *Haemoproteus-Plasmodium* parasites in the Java Sparrow, Javan Munia and White-capped Munia, two unique lineages of *Plasmodium* and *Haemoproteus* respectively [9,10].

This paper reports the finding of prevalence level of avian malaria in the wild bird the coastal area Java island of Indonesia.

Materials and Methods

Sample. In total, 112 DNA samples of 22 species were used to assess the prevalence level of avian malaria in the wild birds (Table 1). In addition domestic chicken and duck, respectively 10 samples were included in this study. The DNA was extracted from the blood of trapped birds at Trisik Beach of Yogyakarta, Java (Indonesia) in 2009. Approximately 25 µl whole blood sample was collected by venipuncture from each bird and preserved either in Queen's lysis buffer [11], ethanol (95%) or FTA. The DNA extraction method used were a standard phenol-chloroform extraction (PCE) protocol [12] or using the DNeasy® Tissue Kit (Qiagen Pty Ltd). In the PCE protocol samples were digested with proteinase K (10–40 mg/mL) in an extraction buffer at 37şC overnight. Purification of DNA was carried out with one extraction with phenol:chloroform: isoamyl alcohol (24:24:1) wash and one extraction with chloroform-isoamyl alcohol (24:1) wash. Precipitation of DNA was done with 2 volumes of absolute ethanol, followed by a washing step in 70% ethanol. DNA was then resuspended in TE buffer (10 mM Tris, 1mM EDTA, pH 7.2). Meanwhile, the second protocol followed the recommended protocol for animal blood (Qiagen Pty Ltd).

PCR amplification. To detect the occurrence of blood parasite, the DNA was amplified by using a nested-PCR assay developed by Hellgren et al. [13]. The protocol is able parallely to detect three common genera blood parasites: *Haemaproteus*,

Table 1. The blood parasites found in the wild bird at Trisik Beach of Yogyakarta

		N	Blood parasite*	
			Haemopreteus--Plasmodium	*Leucocytozoon*
Wild bird				
Yellow bittern	*Ixobrychus sinensis*	3	3	–
Barred buttonquail	*Turnix suscica tor*	10	–	–
Javan plover	*Charadrius javanicus*	10	–	–
Common sandpiper	*Tringa hypoleucos*	5	–	–
Pintail snipe	*Gallinago stenura*	11	3	1
Great crested-tern	*Sterna bergii*	4	–	–
Spotted-dove	*Streptopelia chinensis*	3	–	–
Plantive cuckoo	*Cacomantis merulinus*	1	–	–
Horsfield's bronze cuckoo	*Chrysococcyx basalis*	4	1	–
Savannah nightjar	*Caprimulgus affinis*	11	–	–
Cave-swiftlet	*Collocalia linchi*	14	–	–
Small blue kingfisher	*Alcedo coerulescens*	1	–	–
Javan kingfisher	*Halcyon cyanoventris*	1	–	–
Yellow-vented bulbul	*Pycnonotus goiavier*	1	–	–
Common tailorbird	*Orthotomus sutorius*	1	–	–
Ashy tailorbird	*Orthotomus ruficeps*	1	–	–
Olive-backed pipit	*Anthus hodgsoni*	1	–	–
Long-tailed shrike	*Lanius schach*	4	–	–
Plain-throated sunbird	*Anthreptes malacensis*	1	–	–
Olive-backed sunbird	*Cinnyris jugularis*	8	1	1
Eurasian tree sparrow	*Passer montanus*	10	–	–
Javan munia	*Lonchura leucogastroides*	7	1	0
	Total	112	9	2
Domestic bird				
Chicken	*Gallus gallus domesticus*	10	–	–
Domestic duck	*Anas platyrhynchos domesticus*	10	1	–
	Total	20	1	–

N– number of sample; *– number indicate the number of infected sumples.

Table 2. The lienage of avian malaria in the wild bird and domestic duck at Trisik Beach of Yogyakarta

Species	Blood parasites						
	Plasmodium sp.					*Haemoproteus* sp.	*Leucocytozoon* sp.
	PTRIS1	PTRIS2	PTRIS3	PTRIS4	PTRIS5	HTRIS1	LTRIS1
Pintail snipe (*Gallinago stenura*)		3					1
Horsfield's bronze cuckoo (*Chrysococcyx basalis*)						1	
Olive-backed sunbird (*Cinnyris jugularis*)						1	1
Javan munia (*Lonchura leucogastroides*)			1				
Yellow bittern (*Ixobrychus sinensis*)				1	2		
Domestic duck (*Anas platyrhynchos domesticus*)	1						

Plasmodium, and *Leucocytozoon*. The protocol contains two steps PCR. The first step amplify the cytochrome b (*cyt-b*) of these three genera. The PCR was included ~50 ng of total DNA, 1.25 mM of each deoxynucleoside triphosphate, 1.5 mM $MgCl_2$, 0.6 mM of each primer, and 0.5 units Tag DNA polymerase, and was performed in volume of 25 µl. The primers used were HaemNFI (5'-CATATATTAAGAGAAITATGGAG-3') and Haem NR3 (5'-ATAGAAAGATAAGAA ATACCATTC-3'). PCR was run for 20 cycles in following condition: 94°C for 30 sec., 50°C for 30 sec., and 72°C for 45 sec. The samples were incubated before cyclic reaction at 94°C for 3 min. and after cyclic reaction at 72°C for 10 min.

The second step PCR was used the product of the first step as template on two separate reactions, respectively 1 µl for *Haemoproteus* spp.-*Plamodium* spp. and for *Leucocytozoon* spp. The primers used to amplify the former parasites were HaemF (5'-ATGGTGCTTTCGATATATGCATG-3') and Haem R2 (5'-GCATTATCTGGATGTGATAATGGT -3') [2]. Meanwhile, the primers for the latter were HaemFL (5'-ATGGTG TTTTAGATACTTACAT T-3') and HaemR2L (5'-CATTATCTGGATGAGATA ATGGIGC-3') [13]. These reaction were run separately in the volume of 25µl with the same amount of reagents as in the first step PCR. The thermal condition of the PCR was as the first PCR except for the extent of cycles (35 cycles).

The final PCR products were visualized with agarose gel electrophoresis, by loading 5 ml of the products and 2 ml of loading dye (Bromophenol Blue) onto a 1.2% agarose gel. DNA stain (SYBR Safe) was included onto the gels to visualize the DNA. Gels were run in x1 TBE buffer at 100 mA for approximately 25 minutes.

The positive samples then were selected for sequencing either using primer HaemF (for *Haemoproteus* spp.-*Plamodium* spp.) or HaemFL (for *Leucocytozoon* spp.). Double strand PCR products were purified by ethanol precipitation or spin column purification (Ultra Clean Tm, MO BIO Inc), prior to cycle sequenced using DYEnamic ET Dye Terminator Kit (MegaBACE). Sequencing products were purified and screened using MegaBACE™ DNA Analysis Systems. Identification of parasites were determined by searching for similar sequences through the NCBI's database (http://www.ncbi.nlm.nih.gov/blast/).

Results

In total PCR amplification of cyt-b mtDNA of birds at Trisik Beach positively detected 11 out of 112 samples (9.8%) of all the blood parasites. Only 5 species out of 22 wild bird species were infected by the avian malaria parasites (Table 1). Meanwhile, only one out of 20 samples of domestic birds was infected. All positive samples sequenced consistently generated around 450 base pair nucleotides. Alignments of 12 sequences have revealed six

parasite lineages in the wild bird at Trisik Beach of Yogyakarta, consist of four lineages for *Plasmodium* sp. and the rest respectively one lineages for *Haemoproteus* sp. and *Leucocytozoon* sp. One lineage of *Plasmodium* was found in domestic duck (Tab. 2).

Discussion

This study found that the prevalence of avian malaria infection in the wild bird at Trisik Beach was lower compare to those of the forest bird in Java [8]. Using blood smear method, the latter study found that among 27 bird species of 152 birds assayed the prevalence of infection were between 4.3–17% and 0–0.4%, respectively for *Haemoproteus* and *Plasmodium*. These findings concordances with the previous works that birds living in lowland forests seem to be more susceptible to malaria infection [14].

Using the molecular approach, Ishtiaq et al. [14] found that the estrildid finch in India were not infected with *Haemoproteus* sp. The study which was conducted in three sites in Asia, i.e. India, Myanmar and Korea, also sugested the low level prevalence of the avian malaria in 16 bird families examined, ranged from 0% (Meropidae, Motacillidae, Phasianidae, Picidae, Paradoxirnithidae and Timaliidae) until 26% (Corvidae). On the other hand, the level prevalence of *Plasmodium* was hingger. Parasite *Plasmodium* infected 27.6% birds of Estrildid in India. For other bird families, the prevalence level ranged from 8% (Paradoxirnithidae) untill 50% (Paridae). Another regional study on this subject has been done in the tropical area of Australo-Papuan [15]. The study examined 80 bird species of 8 families. In total, 176 induvidual with 376 birds were infected by blood parasites. The level of prevalence of *Plasmodium* ranged from 3% (Petroicidae) till 47% (Ptilinorynchidae), and the prevalence of *Haemoproteus* ranged from 11.3% (Acanthizidae) till 56% (Petroicidae).

It has been suggested that there are strong host-family specificity in *Haemoproteus* and the lineages of *Plasmodium* are more likely to form evolutionarily-stable associations with novel hosts [15]. The results of this study provide additional evidences for *Plasmodium* lineages that uniquely were only infected the Pintail snipe, Javan Munia. Yellow Bittern and domestic duck. Yet, to get the picture of host specificity of *Haemoproteus* and *Leucocytozoon*, it is necessary to increase the scale of the study, either in the number of bird species or family or its geographical range.

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