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Double trouble: untangling mixed sequence signals in bird samples with avian haemosporidian co-infections

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Abstract

Blood parasites comprise some of the most prevalent pathogens in nature, and their detection and identification are major objectives in varied fields such as ecology and biomedicine. Two approaches were compared, one based on Sanger sequencing and the other next-generation sequencing (NGS) based, in terms of their performance in detecting avian blood parasites across tropical Southeast Asian birds. Across a panel of 528 bird individuals, 43 birds were ascertained to be infected with avian haemosporidians using a polymerase chain reaction-based detection method. Among these samples, NGS-based barcoding confirmed co-infections by multiple blood parasites in all eight cases where Sanger sequencing produced double peaks. Importantly however, the NGS-based method produced another five diagnoses of co-infections (62.5%) in which Sanger-based barcoding remained equivocal. In contrast to Sanger sequencing, the NGS-based method was able to identify co-infecting haemosporidian lineages *via* their barcodes. The accuracy of avian haemosporidian lineage identification was not compromised by the shorter length of NGS sequences, with ~94% of NGS barcodes producing matches identical to those of the Sanger barcodes. The application of NGS-based barcoding methods promises to enhance parasite identification and reduce erroneous inferences based on artefacts.

Introduction

Avian haemosporidians (Apicomplexa: Haemosporida) belonging to the genera *Plasmodium*, *Haemoproteus* and *Leucocytozoon* are diverse blood parasites affecting birds worldwide (Valkiūnas, 2005). Bird hosts infected with avian haemosporidians can suffer from anaemia, weight loss and sickness (Merino *et al.*, 2000; Palinauskas *et al.*, 2008, 2018) and be subject to high mortality rates in susceptible populations. Avian infections can be common, with many bird individuals tolerating chronic infections for life after surviving the acute malaria phase (Asghar *et al.*, 2011). Such infections can have significant effects on life history traits and may act as selective agents in wild bird populations (Asghar *et al.*, 2011).

Avian haemosporidians spend part of their life cycles in bird hosts and dipteran vectors, with highly interdependent ecological relationships. Therefore, accurate detection of avian haemosporidians is important in addressing questions regarding host–vector–parasite interactions, ecology and evolution (Santiago-Alarcon *et al.*, 2012; Sehgal, 2015; Pacheco *et al.*, 2018*a*). Moreover, accurate detection is also needed for understanding the epidemiology of infections and developing disease and vector control strategies, especially in places where avian conservation is of concern.

The detection of blood parasites can be challenging. Accurate identification of avian haemosporidians from blood smears is difficult as it requires a high level of taxonomic expertise to differentiate minute characters across taxa while accounting for the range of morphological features resulting from various developmental stages (Valkiūnas *et al.*, 2014).

Since the 2000s, molecular techniques have been widely used in the detection and characterization of avian blood parasites, including a widely used nested polymerase chain reaction (PCR) protocol to amplify a fragment of the mitochondrial cytochrome b (cytb) gene (Bensch *et al.*, 2000; Hellgren *et al.*, 2004). The amplified products are subsequently sequenced *via* the traditional Sanger approach and are used as the barcode for lineage identification by matching identified sequences available in the public MalAvi database (Bensch *et al.*, 2009). A recently published protocol incorporates the use of three different primer sets targeting *Haemoproteus*, *Plasmodium* and *Leucocytozoon*, respectively, in a multiplex PCR (Ciloglu *et al.*, 2019). The resulting amplicons yield products of different size for each genus, allowing for rapid genus-level identification. However, the current repertoire of molecular methods does not allow for haemosporidian lineage identification in the amplified products from bird individuals co-infected by multiple parasite lineages belonging to the same genus. In capillary sequencing outputs, amplified genes from co-infected individuals often show up as jumbled chromatograms, sometimes with double peaks (‘double base calling’) (Marzal *et al.*, 2008), making it virtually impossible to distinguish blood parasite strains apart.

As it is difficult to recover fully from parasitic infections, and considering the relatively long lifespan of birds, co-infections with multiple blood parasite strains are thought to be common

in wild birds, although rarely detected (Valkiūnas, 2005; Bensch *et al.*, 2007). Crucially, co-infections by multiple parasites of the same haemosporidian genus may be more common than multigeneric mixed infections (Silva-Iturriza *et al.*, 2012). In cases where multiple blood parasite infections are suspected due to the presence of double peaks, the fragments are usually subjected to an additional tedious cloning step and separately sequenced (Pérez-Tris and Bensch, 2005; Silva-Iturriza *et al.*, 2012). The presence of co-infections can also be ascertained by examining blood smear slides of the affected bird hosts (Valkiūnas *et al.*, 2014), although many co-infections are overlooked this way (Xuan *et al.*, 2021). Additionally, if blood samples are stored exclusively in buffers for molecular study, verification of co-infected samples *via* microscopy is no longer possible as blood smear slides cannot be prepared.

With the advance of next-generation sequencing (NGS), high-throughput sequencing of heterogeneous barcodes can be carried out simultaneously at a fraction of the cost of Sanger sequencing (Meier *et al.*, 2016; Yeo *et al.*, 2020). Employing NGS methods in the avian haemosporidian screening process provide an added advantage of retrieving multiple parasite lineages in co-infected birds. Even so, to the best of our knowledge, NGS has not been applied to avian haemosporidian detection and identification.

In the present study, a PCR assay and workflow suitable for avian haemosporidian screening on an NGS Illumina platform was developed. The detection rates of this assay were compared against detections using the first primer pair (HaemNF1 and HaemNR3) of the widely used nested PCR protocol (Hellgren *et al.*, 2004), and sequence similarities of the cytb barcodes recovered from both methods were evaluated, allowing for an assessment of the reliability of the NGS method in detecting co-infections. The potential implications of previous practices of exclusively relying on Sanger sequencing were discussed, followed by a critical evaluation of the accuracy of using short NGS barcodes as compared to longer Sanger reads in avian haemosporidian identification.

Materials and methods

Sample selection

Since 2013, collections of avian blood and tissue (pectoral muscle) samples have been ongoing in Singapore, and a substantial collection has been amassed. Blood samples have mostly been obtained from mist netting at various locations under requisite permit, while tissue samples have been obtained through subsampling bird carcasses submitted to our laboratory by the public or by various agencies and organizations (see Acknowledgements for details). These bird carcasses are exclusively the result of window strikes or death by natural causes. Tissue samples were included in the screening as they have been found to be as reliable as blood samples in the detection of avian haemosporidians (Fecchio *et al.*, 2019). In addition to the Singaporean samples, a smaller number of blood samples from Brunei (Sadanandan *et al.*, 2015), Cambodia, Laos, Indonesia (Rheindt *et al.*, 2020), Malaysia and Vietnam – all collected for previous projects – were also used (Appendix 1; see Acknowledgements for lending institutions). All samples used in this study were preserved directly in molecular-grade absolute ethanol and stored at −20°C. To ensure screening of a wide range of species for avian haemosporidians, we selected birds from across various orders for which there was a minimum of eight unique individual samples available, making exceptions for rare species or species of particular interest. A total of 528 individuals representing 58 bird species across 17 orders were screened (Appendix 1).

Primer design

To enable the detection and sequencing of multiple avian haemosporidian strains in a co-infected blood sample *via* NGS, a reverse primer was designed (HaemNRShort, 5′-GATTAG AGCTACCTTGTAAATGTA-3′). To do so, 114 cytb sequences across a wide range of *Haemoproteus*, *Plasmodium*, *Leucocytozoon* and *Hepatocystis* strains (Pacheco *et al.*, 2018*b*) were downloaded and a sufficiently conserved region was targeted. The Oligonucleotide Properties Calculator (http://biotools.nubic.northwestern.edu/OligoCalc.html) was used to ensure that the designed primer satisfies standard requirements such as optimal primer length, GC content and lack of potential hairpin formation. The primer sequence was then checked against cytb sequences of avian species to preclude cross-amplification. This new primer, in combination with a widely used published forward primer (HaemNF1; Hellgren *et al.*, 2004), produces a cytb barcode fragment of 367 base pairs, which is suitable for sequencing on an Illumina NGS platform (Fig. 1). In order to sequence the barcodes on an NGS platform and demultiplex them successfully in the bioinformatic stage, a one-step PCR reaction with unique primer-tag combinations following Meier *et al.* (2016) was implemented.

Molecular work

DNA extractions were carried out with the DNeasy® Blood & Tissue Kit (Qiagen, GmbH, Hilden, Germany) to obtain genomic DNA from the avian samples. To screen and detect avian haemosporidians using the traditional approach, PCR was carried out. Positive and negative controls were included in every batch of PCR. The reaction volumes of 25.0 μL were set up as follows: 2.5 μL 10× Taq Buffer with $(NH_4)_2SO_4$, 2 mM $MgCl_2$, 0.2 mM dNTP Mix, 0.0002 mM of each primer (Sanger primer pair), 0.625 units of DreamTaq DNA polymerase, at least 5 ng of DNA template and sterilized millipore water to make up the volume. An initial denaturation step (94°C, 3 min), 30 cycles of denaturation (94°C, 30 s), annealing (50°C, 40 s) and extension (72°C, 1 min) and a final extension step (72°C, 10 min) were carried out using the Mastercycler nexus gradient (Eppendorf, Hamburg, Germany). To ensure repeatability and to ascertain if there were any false-negative or false-positive results, triplicate screening with the Sanger primer pair was conducted for 40 randomly selected samples (including samples without avian haemosporidian infections). Subsequently, amplification with the NGS primer pair was carried out for the samples which had tested positive for avian haemosporidians on the basis of the Sanger primer pair. Tests for haemosporidian infection were considered positive when a band was seen in a 2% agarose gel after visualization with a UV transilluminator (Syngene, Synoptics Limited, Cambridge, UK).

Samples amplified with the Sanger primer pair were cleaned up using ExoSAP-IT® and cycle-sequenced with the BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems Inc., Foster City, CA, USA). Sequencing was carried out on an Applied Biosystems 3130XL Genetic Analyzer. Samples amplified with the NGS primer pair were pooled in equimolar proportions and sequenced on an Illumina MiSeq 300 bp paired-end platform (Novogene, Beijing, China).

Bioinformatics

Sanger sequences were examined and edited using CodonCode™ Aligner version 8.0.2 (LI-COR, Inc., Lincoln, NE, USA) for ends trimming, resolution of ambiguous bases and to assemble contigs from forward and reverse strands. Double peaks (double base

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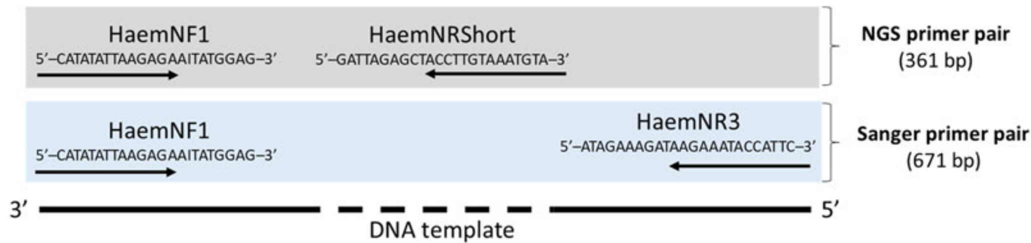

Fig. 1. Schematic illustration of the directions and combinations of the two different primer pairs used for mitochondrial cytochrome b gene amplification in this study. The next-generation sequencing (NGS) primer pair consists of the forward primer HaemNF1 and the newly designed reverse primer HaemNRShort. The Sanger primer pair (HaemNF1 and HaemNR3) was designed by Hellgren *et al.* (2004).

calling) in the sequences were scored as such when they occurred in both the forward and reverse strand chromatograms at the same base pair positions, and were replaced with corresponding ambiguity codes.

Quality checks for the Illumina raw read files were carried out using FastQC (Andrews, 2010), and adaptors were removed using Cutadapt (Martin, 2011). Demultiplexing and subsequent steps such as dereplication, denoising and taxonomic assignment were carried out using the OBITools suite which has been optimized for metabarcoding (Boyer *et al.*, 2016). The final output files contained unique sequences obtained from each sample with their respective counts.

To avoid incorporating possible contamination in downstream analysis, only haemosporidian sequences of the top two highest counts (referred to as dominant and subdominant sequences subsequently) were analysed for each bird individual. A ratio of subdominant sequence count over dominant sequence count was calculated across all the positively infected bird samples. Samples with low coverage (dominant count lower than 1000) were excluded. The resulting cytb sequences were aligned using MAFFT version 7 (Katoh and Standley, 2013). To determine if sequences obtained from both Sanger and NGS platforms match, they were visualized in AliView (Larsson, 2014) and bases with ambiguity codes within the Sanger sequences were cross-examined against NGS sequences at the same base positions.

To check for the presence of pseudogenes, all cytb sequences were translated into amino acid sequences and the reading frames were permutated to check for the presence of stop codons in AliView. To assess sequence divergence, pairwise *p*-distances between the Sanger and the dominant and subdominant NGS sequences, respectively, were calculated with MEGA11 (Tamura *et al.*, 2021).

Comparing the performance of short and long cytb sequences in lineage identification

To compare the accuracy of lineage identification with short NGS sequences (~330 bp), all Sanger and NGS cytb sequences generated were matched against curated sequences from the global avian haemosporidian MalAvi database (Bensch *et al.*, 2009). Among the NGS sequences, only dominant barcodes were included in the analysis, except in cases where the subdominant NGS sequence matched the Sanger sequence (D2179, D2003 and F1102). From here onwards, we referred to them as 'homologous NGS sequences'. The Basic Local Alignment Search Tool (BLAST) v2.12 (Altschul *et al.*, 1990) was used at an expect value (*e*-value) cut-off of 1×10^{-5}. A comparison of the top identity match(es) (%), sequence length and number of mismatches between the NGS and Sanger sequences was carried out. If possible, species names were assigned to perfect matches when such taxonomic information was available in MalAvi. In most other cases, the cytb barcodes were assigned the so-called lineage names. Lineages on the MalAvi database are defined based on the currently implemented 'one base pair rule' method, whereby a single base pair difference in the cytb barcode is interpreted as species-level differentiation (Galen *et al.*, 2018). Such practice may lead to an overestimation of avian haemosporidian diversity (Hellgren *et al.*, 2014; Palinauskas *et al.*, 2017; Galen *et al.*, 2018). Therefore, a more conservative approach was taken in this case, considering variations of up to two base pair differences as the same lineage (Martinsen *et al.*, 2006; Ricklefs *et al.*, 2014).

Results

Avian haemosporidian detection with Sanger primer pair

Out of 528 bird samples screened, a total of 43 were positive for avian haemosporidians (8.14%) on the basis of gel electrophoresis. All PCR triplicates of 40 samples that had been randomly selected from a total of 528 samples produced consistent results, with the exception of one sample (M1622) (97.5%) (Appendix 2). This outcome indicated that screening is highly repeatable, with a low incidence of false positives or negatives. Out of the 43 positive samples, two were not sequenced successfully (L2344 and L2339) on the Sanger sequencing platform as the chromatograms were jumbled.

Examination of the Sanger chromatograms of successfully sequenced individuals revealed that eight samples (19.0%) contained double peaks in their cytb chromatograms (Fig. 2, Table 1), indicating the likely presence of co-infecting avian haemosporidian strains. Among them, A/G type double peaks were the most common (Table 1).

Avian haemosporidian detection with NGS primer pair

The newly developed NGS primer was successful in the detection of avian blood parasites: all samples testing positive with the Sanger primer set were successfully amplified with the NGS primer set as well. Two samples (L2344 and JBP121; Table 1) were excluded from downstream analysis due to low coverage (<1000 times coverage for the dominant sequence).

The cytb sequences obtained from the Sanger method always matched with either the dominant or subdominant NGS sequences, even in cases where the Sanger double peak is not reflected fully in the NGS sequences. In most cases (92.7%), the Sanger sequences matched with the dominant NGS sequence (Fig. 3; Table 1, last column). No stop codons were found in all the sequences.

Detection of co-infections using both methods

Based on the comparison of Sanger and NGS-based detection patterns, we considered a subdominant to dominant NGS sequence ratio of 0.2 to be the lower limit at which haemosporidian co-infections can be conclusively diagnosed (Fig. 3). For samples

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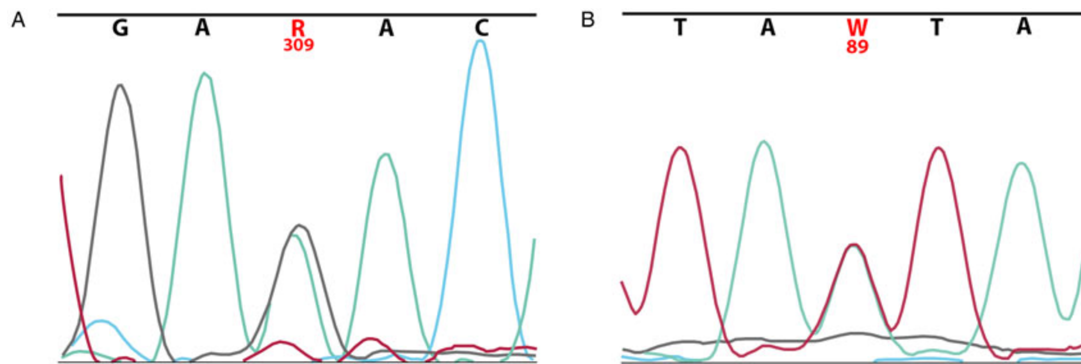

Fig. 2. Two examples of double peaks detected in the chromatograms of the Sanger sequences of a single individual. (A) An A/G (green and black) double peak at position 309 and (B) an A/T (green and red) double peak at position 89.

with ratios below 0.2, we only considered their dominant sequences as representative of the infecting haemosporidian in order to avoid incorporating contaminant sequences in downstream analysis. A total of 13 samples were found to be co-infected using this NGS-based threshold (Fig. 3, Table 1). Among these samples, eight exhibited double peaks in the Sanger chromatograms. Four samples did not show signs of double peaks and the one remaining sample exhibited a jumbled, illegible trace chromatogram (L2339). The ambiguity codes of Sanger double peaks of two samples did not fully match the dominant and subdominant NGS sequences at the same base positions (Fig. 3, Table 1). For example, in sample M1866, a Sanger double peak of A/G was detected, but both dominant and subdominant NGS sequences only reflected G at that base position.

Among the eight co-infected samples containing double peaks in the chromatograms, a large majority (7) exhibited a signature of mixed infections involving highly similar haemosporidian lineages based on the detection of *p*-distance sequence divergences of <1% between the dominant and subdominant NGS sequences. In contrast, the haemosporidian lineages involved in the remaining five mixed infections (detected through the NGS method alone) were less similar.

Comparing lineage identification performance between short NGS and longer Sanger reads

Samples which were not successfully Sanger or NGS sequenced (3) were excluded from this analysis. Similarly, cases in which a Sanger sequence produced multiple equal top BLAST hits to the MalAvi database (7) were also excluded. An overwhelming proportion of homologous NGS cytb barcodes exhibited an identical match to those of the Sanger barcodes (93.93%) (Table 2). Only in two instances, having a longer Sanger sequence improved the accuracy of lineage identification whereas the shorter homologous NGS sequences produced ambiguous BLAST matches.

Discussion

The detection and lineage identification of avian haemosporidians has long been challenging, even more so in cases of mixed infections. Mixed infections may not always manifest themselves as 'double peaks' in Sanger chromatograms and are reported to be highly underestimated (Valkiūnas *et al.*, 2014). In this study, a PCR assay suitable for use with an NGS platform (Illumina) was developed and a metabarcoding workflow was employed to allow for the detection of multiple avian haemosporidian lineages within individual birds. The NGS workflow reflected the same number of infections as the Sanger workflow, but detected 62.5% more cases of co-infections based on the 0.2 threshold applied to subdominant over dominant sequence count ratios.

The 0.2 threshold applied here is based on a conservative evaluation of subdominant to dominant NGS sequence count ratios (Fig. 3). Among the samples which exhibited double peaks in the Sanger chromatograms, the lowest subdominant to dominant NGS sequence count ratio was 0.24. Naturally, applying a cautious threshold of 0.2 might mean missing out on mixed infections where the parasitaemia of the secondary (or even tertiary) co-infection is substantially lower than the primary infection. In fact, the detection of multiple infections with a ratio of less than 0.2 which had a match between Sanger sequence and subdominant sequence points to such instances of co-infection (Table 1). The detection of such mixed infections is challenging because it is difficult to differentiate low secondary parasitaemia from background sequence contamination. Unfortunately, as the samples used here were stored directly in molecular-grade ethanol, we were unable to determine the level of parasitaemia in the infected individuals on the basis of blood smear slides. Hence, our identification of 13 co-infections among 43 total infections must be considered a conservative estimate.

With the exception of one case (D2003) in which double peaks were observed in the Sanger chromatograms, the sequence divergences of the dominant and subdominant sequences in co-infected cases were less than 1% (Table 1). In contrast, the majority of co-infections that were identified through NGS sequencing alone involved species with less similar sequence profiles (>1.5% *p*-distance). This pattern suggests that mixed infections involving dissimilar haemosporidian lineages are more likely to be overlooked when Sanger PCR assays are used, or are discarded as uninterpretable mixed traces (Tenney *et al.*, 2007; Dmitriev and Rakitov, 2008).

The newly developed NGS assay based on Illumina short reads yields shorter fragments (330+ bp) compared to the widely used Sanger primer pair which produces cytb fragments of around 470 bp, with unknown effects on lineage identification accuracy. In the present study, the NGS barcodes largely converged on the same haemosporidian species or lineages when compared to Sanger barcodes. Shorter barcodes may sometimes produce ambiguous identifications (multiple equal matches) in instances where longer Sanger barcodes are able to yield a more precise lineage identification, although this occurred rarely (6.06%) in the present study. Shorter barcodes have been shown to exhibit a higher amplification success rate across a wide range of animal and plant taxa (Meusnier *et al.*, 2008; Françoso and Arias, 2013; Little, 2013), and this may provide an added advantage in detecting avian haemosporidians at low parasitaemia. Future studies should rigorously test the performance of NGS barcodes by comparing them with full-length cytb barcodes *in silico* and determine

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Table 1. Summary of 43 blood samples testing positive for avian haemosporidian infection on the basis of gel electrophoresis, including details on cytochrome b sequences obtained from next-generation sequencing (NGS) (grey) and from Sanger sequencing (blue)

	Bird host			NGS sequences			Sanger sequences	Sequence divergence (%)	
Sample	Common name	Species	Locality sampled	Dominant sequence count (*D*)	Subdominant sequence count (*S*)	Ratio *S*/*D*	Type of double peaks (no.)	Between dominant and subdominant NGS sequences	Between Sanger and dominant NGS sequences
M1868	Oriental magpie-robin	*Copsychus saularis*	Singapore	2353	1964	0.83	–	0.552	0
D2204	Collared kingfisher	*Todiramphus chloris*	Singapore	2595	2091	0.81	G, A (5)	0.829	0
D2590	Collared kingfisher	*Todiramphus chloris*	Singapore	4609	3502	0.76	G, A (5)	0.829	0
L2328	White-rumped shama	*Copsychus malabaricus*	Singapore	7112	4971	0.70	–	3.039	0
M1866	Oriental magpie-robin	*Copsychus saularis*	Singapore	2704	1651	0.61	G, A (1)[a] T, A (1)	0.276	0
D2201	Collared kingfisher	*Todiramphus chloris*	Singapore	8005	4622	0.58	G, A (4)	0.829	0
D2206	Collared kingfisher	*Todiramphus chloris*	Singapore	3707	2046	0.55	G, A (5)	0.829	0
D2179	Collared kingfisher	*Todiramphus chloris*	Singapore	8052	4083	0.51	–	1.657	1.681
CR492	Blue-winged pitta	*Pitta moluccensis*	Singapore	3584	1811	0.51	–	8.564	0
D2802	Collared kingfisher	*Todiramphus chloris*	Singapore	4922	2321	0.47	G, A (4)[a]	0.829	0
M1867	Oriental magpie-robin	*Copsychus saularis*	Singapore	3217	1200	0.37	G, A (3)	0.553	0
L2329	White-rumped shama	*Copsychus malabaricus*	Singapore	1658	537	0.32	No sequence	3.040	NA
D2003	Collared kingfisher	*Todiramphus chloris*	Singapore	4553	1093	0.24	G, A (1)	1.657	1.515
J4163	Siberian blue robin	*Larvivora cyane*	Singapore	11912	1914	0.16	–	7.459	0
D1907	Collared kingfisher	*Todiramphus chloris*	Singapore	8816	947	0.11	–	0.829	0
AV01	Rock pigeon	*Columba livia*	Singapore	13249	1373	0.10	–	0.829	0
F0822	Pink-necked green pigeon	*Treron vernans*	Singapore	2246	187	0.08	–	1.393	0
D9439	Collared kingfisher	*Todiramphus chloris*	Singapore	8242	663	0.08	–	1.657	0
D2205	Collared kingfisher	*Todiramphus chloris*	Singapore	12755	900	0.07	–	0.828	0
M1622	Asian glossy starling	*Aplonis panayensis*	Singapore	3125	217	0.07	–	9.945	0
JBP144	Brown hawk owl	*Ninox scutulata*	Singapore	11076	662	0.06	–	1.657	0
AVA632RBC	Brown hawk owl	*Ninox scutulata*	Singapore	7165	374	0.05	–	1.657	0
F1102	Sunda scops owl	*Otus lettia*	Singapore	1274	50	0.04	–	2.486	0.326
CR025	Pink-necked green pigeon	*Treron vernans*	Singapore	1768	67	0.04	–	2.210	0
D2208	Collared kingfisher	*Todiramphus chloris*	Singapore	9246	335	0.04	–	1.657	0
D2556	Collared kingfisher	*Todiramphus chloris*	Singapore	14636	516	0.04	–	1.657	0
WLK606	Northern boobook	*Ninox japonica*	Singapore	6706	196	0.03	–	1.657	0

(*Continued*)

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Table 1. (*Continued.*)

	Bird host			NGS sequences			Sanger sequences	Sequence divergence (%)	
Sample	Common name	Species	Locality sampled	Dominant sequence count (*D*)	Subdominant sequence count (*S*)	Ratio *S*/*D*	Type of double peaks (no.)	Between dominant and subdominant NGS sequences	Between Sanger and dominant NGS sequences
AVA611RBC	Spotted wood owl	*Strix seloputo*	Singapore	4369	127	0.03	–	2.486	0
F0980	Pink-necked green pigeon	*Treron vernans*	Singapore	10243	276	0.03	–	3.039	0
D1810	Collared kingfisher	*Todiramphus chloris*	Singapore	3264	75	0.02	–	1.657	0
OWBM82083	Oriental magpie-robin	*Copsychus saularis*	Malaysia (Sarawak)	7753	206	0.03	–	7.459	0
D2560	Collared kingfisher	*Todiramphus chloris*	Singapore	13060	298	0.02	–	1.657	0
F0760	Pink-necked green pigeon	*Treron vernans*	Singapore	1453	32	0.02	–	1.657	0
D2561	Collared kingfisher	*Todiramphus chloris*	Singapore	10677	227	0.02	–	0.829	0
D2592	Collared kingfisher	*Todiramphus chloris*	Singapore	5409	101	0.02	–	0.829	0
Ninox_Brunei	Northern boobook	*Ninox japonica*	Brunei	10302	206	0.02	–	0.829	0
CR522	Spotted wood owl	*Strix seloputo*	Singapore	7045	131	0.02	–	8.840	0
D2582	Collared kingfisher	*Todiramphus chloris*	Singapore	8191	137	0.02	–	2.210	0
D2576	Collared kingfisher	*Todiramphus chloris*	Singapore	8869	129	0.01	–	2.486	0
D2585	Collared kingfisher	*Todiramphus chloris*	Singapore	8001	95	0.01	–	0.829	0
D2579	Collared kingfisher	*Todiramphus chloris*	Singapore	4558	51	0.01	–	1.657	0
L2344	White-rumped shama	*Copsychus malabaricus*	Singapore	77	57	0.74	No sequence	1.660	NA
JBP121	Lineated barbet	*Megalaima lineata*	Singapore	310	29	0.09	–	10.221	0

For NGS data, counts are shown for dominant (*D*) and subdominant (*S*) sequence counts only. Samples are ordered according to their count ratio, as calculated by dividing subdominant sequence counts over dominant sequence counts (*S*/*D*). The thick black line indicates the 0.2 ratio threshold used, with samples above the line considered to be co-infected. Sequence divergences were calculated using the *p*-distance method. Samples highlighted in yellow were excluded from downstream analyses owing to low sequence coverage (<1000× in dominant NGS sequence count).

[a]Sanger double peaks in which one out of the two called bases does not match the dominant or subdominant NGS sequences.

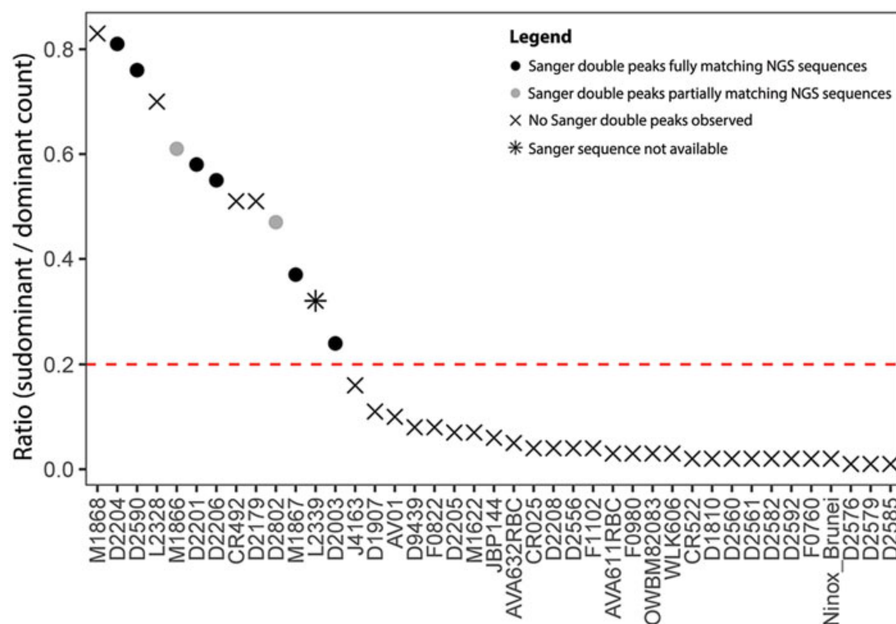

Fig. 3. Descending ratio of subdominant next-generation sequence (NGS) counts over dominant NGS counts, with information on the presence or absence of congruence between NGS and Sanger sequences. A total of 41 samples which were successfully amplified (showing up as positive in gel electrophoresis) and which exhibited >1000× in the dominant NGS sequence count are shown here. The red dotted horizontal line indicates the 0.2 ratio threshold used, and samples above the line are considered co-infected.

Table 2. A comparison of the Basic Local Alignment Search Tool (BLAST) results of short NGS and longer Sanger barcodes when matched against the global avian haemosporidian (MalAvi) database

Category	Description	Frequency
Correct	Top BLAST hit of homologous NGS sequence exhibits exact match to that of Sanger sequence	25
	Homologous NGS sequence exhibits multiple equal top BLAST hits matching the same lineage as Sanger top BLAST hit	6
Ambiguous	Homologous NGS sequence exhibits multiple equal top BLAST hits, one of which does not match the same lineage as the Sanger top BLAST hit	2
Excluded	Sanger sequence with multiple equal top BLAST hits	7
	Sanger sequencing not successful	3

Frequency refers to the number of samples matching the description.

if the shorter barcodes are informative across positions and lengths.

Based on the screening results, only congeneric infections were detected. The widely used nested PCR protocol often preferentially amplifies *Haemoproteus* DNA in mixed infections of *Plasmodium* and *Haemoproteus* (Bernotienė *et al.*, 2016; Ciloglu *et al.*, 2019). Future studies can be carried out to test the NGS assay against samples which have been screened morphologically for blood parasites, or against samples with known parasitaemia loads.

This NGS assay has several advantages over currently available detection methods and exhibits improvements in detecting mixed infections when compared with Sanger sequencing. The use of the NGS assay allows co-infecting haemosporidians within an avian host to be identified effectively. Barcoding on NGS platforms also lowers per-sample sequencing costs when compared to Sanger sequencing (Yeo *et al.*, 2020), especially when screening a large number of samples in the order of hundreds to thousands. Additionally, shorter barcodes have been shown to amplify significantly better than longer barcodes (Yeo *et al.*, 2020). There are multiple published metabarcoding pipelines that can be employed in analysing such sequences (Edgar, 2010; Boyer *et al.*, 2016; Callahan *et al.*, 2016), further increasing the ease of detecting avian haemosporidians and their mixed infections. This method is also particularly suited for samples stored in appropriate buffers for molecular work, but without available blood smear slides to assess mixed infection status and/or parasitaemia loads. The application of NGS-based barcoding methods can hence enhance parasite identification and reduce erroneous inferences of co-infections based on artefacts in Sanger sequencing, especially when co-infections are a common occurrence.

Data

The cytb barcodes obtained from both Sanger sequencing and the NGS-based method have been uploaded to Genbank (OM649638–OM649760). The full length cytb barcodes have also been deposited in the avian haemosporidian barcode database MalAvi.

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Conflict of interest. None.

Ethical standards. This study received approval from the National University of Singapore (NUS) Institutional Animal Care and Use Committee (B16-00572) and laboratory work was conducted in accordance with NUS's Office of Safety, Health and Environment regulations.

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Appendix 1

List of birds screened and the associated avian haemosporidian infection prevalence

Table A1

Order	Common name	Species	Number screened	Positive	Negative	Country
Galliformes	Red junglefowl	*Gallus gallus*	10	0	10	Singapore
Columbiformes	Rock pigeon	*Columba livia*	14	1	13	Singapore
	Emerald dove	*Chalcophaps indica*	13	0	13	Singapore
	Zebra dove	*Geopelia striata*	1	0	1	Singapore
	Pink-necked green pigeon	*Treron vernans*	15	4	11	Singapore
Cuculiformes	Chestnut-winged cuckoo	*Clamator coromandus*	8	0	8	Singapore
	Asian koel	*Eudynamys scolopacea*	14	0	14	Singapore
Caprimulgiformes	Large-tailed nightjar	*Caprimulgus macrurus*	8	0	8	Singapore
Apodiformes	Black nest swiftlet	*Aerodramus maximus*	2	0	2	Singapore
	Edible nest swiftlet	*Aerodramus fuciphagus*	8	0	8	Singapore
Gruiformes	White-breasted waterhen	*Amaurornis phoenicurus*	8	0	8	Singapore
	Watercock	*Gallicrex cinerea*	8	0	8	Singapore
Charadriiformes	Lesser sand plover	*Charadrius mongolus*	8	0	8	Singapore
	Red-necked stint	*Calidris ruficollis*	8	0	8	Singapore
Ciconiiformes	Milky stork	*Mycteria cinerea*	2	0	2	Singapore
	Painted stork	*Mycteria leucocephala*	3	0	3	Singapore
	Hybrid stork (painted/milky)	–	3	0	3	Singapore
Pelecaniformes	Yellow bittern	*Ixobrychus sinensis*	8	0	8	Singapore
	Dalmatian pelican	*Pelecanus crispus*	8	0	8	Singapore
Accipitriformes	Changeable hawk eagle	*Nisaetus cirrhatus*	8	0	8	Singapore
	Brahminy kite	*Haliastur indus*	8	0	8	Singapore
Strigiformes	Collared scops owl (=Sunda scops owl)	*Otus [lettia] lempiji*	8	1	7	Singapore
	Spotted wood owl	*Strix seloputo*	5	2	3	Singapore
	Brown boobook	*Ninox scutulata*	5	2	3	Singapore
	Northern boobook	*Ninox japonica*	3	1 (B) (S)	1	Brunei, Singapore
Bucerotiformes	Oriental pied hornbill	*Anthracoceros albirostris*	3	0	3	Singapore
Piciformes	Laced woodpecker	*Picus vittatus*	8	0	8	Singapore
	Lineated barbet	*Psilopogon lineatus*	8	1	7	Singapore

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Coraciiformes	White-throated kingfisher	*Halcyon smyrnensis*	8	0	8	Singapore
	Collared kingfisher	*Todiramphus chloris*	38	20	18	Singapore, Indonesia
	Blue-eared kingfisher	*Alcedo meninting*	1	0	1	Singapore
	Black-backed kingfisher	*Ceyx erithaca*	1	0	1	Singapore
Psittaciformes	Red-breasted parakeet	*Psittacula alexandri*	8	0	8	Singapore
	Rose-ringed parakeet	*Psittacula krameri*	8	0	8	Singapore
	Blue-crowned hanging parrot	*Loriculus galgulus*	1	0	1	Singapore
Passeriformes (Suboscines)	Blue-winged pitta	*Pitta moluccensis*	8	1	7	Singapore
Passeriformes (Oscines)	Tiger shrike	*Lanius tigrinus*	8	0	8	Singapore
	Greater racket-tailed drongo	*Dicrurus paradiseus*	13	0	13	Singapore
	House crow	*Corvus splendens*	16	0	16	Singapore
	Olive-winged bulbul	*Pycnonotus plumosus*	34	0	34	Singapore
	Cream-vented bulbul	*Pycnonotus simplex*	1	0	1	Singapore
	Pin-striped tit-babbler	*Mixornis gularis*	16	0	16	Singapore
	Common tailorbird	*Orthotomus sutorius*	8	0	8	Singapore
	Rufous-tailed tailorbird	*Orthotomus sericeus*	8	0	8	Singapore
	Ashy tailorbird	*Orthotomus ruficeps*	21	0	21	Singapore
	Abbott's babbler	*Pellorneum abbotti*	15	0	15	Singapore
	Short-tailed babbler	*Pellorneum malaccensis*	6	0	6	Singapore
	Oriental white-eye	*Zosterops palpebrosus*	8	0	8	Singapore
	Asian glossy starling	*Aplonis panayensis*	8	1	7	Singapore
	Javan myna	*Acridotheres javanicus*	8	0	8	Singapore
	Oriental magpie-robin	*Copsychus saularis*	21	1 (M) 3 (S)	17	Cambodia [MNHN], Indonesia, Laos [MNHN], Malaysia [BM], Singapore, Vietnam [MNHN]
	White-rumped shama	*Copsychus malabaricus*	8	3	5	Singapore
	Siberian blue robin	*Larvivora cyane*	8	1	7	Singapore
	Brown-throated sunbird	*Anthreptes malacensis*	17	0	17	Singapore
	Van Hasselt's sunbird	*Leptocoma brasiliana*	1	0	1	Singapore
	Crimson sunbird	*Aethopyga siparaja*	8	0	8	Singapore
	Little spiderhunter	*Arachnothera longirostra*	9	0	9	Singapore
	Forest wagtail	*Dendronanthus indicus*	8	0	8	Singapore, Indonesia
		Total	528	43	485	

Country where the positive sample was obtained: B, Brunei; M, Malaysia; S, Singapore.
Majority of the samples are housed in the Avian Evolution Lab (National University of Singapore), unless otherwise indicated from lending institutions: BM, Burke Museum; MNHN, Muséum National d'Histoire Naturelle.

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Appendix 2

Triplicate screening results of 40 randomly selected samples

Table A2

Common name	Sample number	PCR 1 results	PCR 2 results	PCR 3 results
Abbott's babbler	L2522	Negative	Negative	Negative
Ashy tailorbird	J4292	Negative	Negative	Negative
Asian glossy starling	M1622	Positive	Negative	Negative
Asian koel	AK038	Negative	Negative	Negative
Brown-throated sunbird	K1313	Negative	Negative	Negative
Collared kingfisher	D2592	Positive	Positive	Positive
Common tailorbird	J4229	Negative	Negative	Negative
Cream-vented bulbul	M2202	Negative	Negative	Negative
Crimson sunbird	J4143	Negative	Negative	Negative
Emerald dove	K0589	Negative	Negative	Negative
Greater racket-tailed drongo	N1425	Negative	Negative	Negative
House crow	HC149	Negative	Negative	Negative
Laced woodpecker	N0914	Negative	Negative	Negative
Lesser sand plover	C0477	Negative	Negative	Negative
Little spiderhunter	K0831	Negative	Negative	Negative
Olive-winged bulbul	M2507	Negative	Negative	Negative
Oriental magpie-robin	M1868	Positive	Positive	Positive
Oriental white-eye	LEI20009	Negative	Negative	Negative
Pin-striped tit-babbler	K1160 L1782	Negative	Negative	Negative
Pink-necked green pigeon	F0822	Positive	Positive	Positive
Red junglefowl	H0035	Negative	Negative	Negative
Rock pigeon	AV01	Positive	Positive	Positive
Rufous-tailed tailorbird	J4085	Negative	Negative	Negative
Short-tailed babbler	L2523	Negative	Negative	Negative
Black-nest swiftlet	BNS1	Negative	Negative	Negative
Black-backed kingfisher	No ID	Negative	Negative	Negative
Blue-crowned hanging parrot	No ID	Negative	Negative	Negative
Blue-eared kingfisher	Q0067	Negative	Negative	Negative
Blue-winged pitta	CR140(a)	Negative	Negative	Negative
Brown boobook	WLK582	Negative	Negative	Negative
Edible-nest swiftlet	ENS1	Negative	Negative	Negative
Forest wagtail	K1133	Negative	Negative	Negative
Javan myna	C1364	Negative	Negative	Negative
Northern boobook	WLK606	Positive	Positive	Positive
Siberian blue robin	J3255	Negative	Negative	Negative
Collared scops owl	H09049	Negative	Negative	Negative
Van Hasselt's sunbird	J3838	Negative	Negative	Negative
White-throated kingfisher	E1404	Negative	Negative	Negative
Zebra dove	N0537	Negative	Negative	Negative

The sample with inconsistent results is highlighted.

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