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Assessing Diet of the Rufous-Winged Philentoma (*Philentoma pyrhoptera*) in Lowland Tropical Forest using Next-Generation Sequencing

(Penilaian Diet Filentoma Sayap Merah (*Philentoma pyrhoptera*) di Hutan Tropika Tanah Rendah menggunakan Penjujukan Generasi Seterusnya)

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ABSTRACT

*Dietary study provides understanding in predator-prey relationships, yet diet of tropical forest birds is poorly understood. In this study, a non-invasive method, next-generation sequencing (Illumina MiSeq platform) was used to identify prey in the faecal samples of the Rufous-winged Philentoma (*Philentoma pyrhoptera*). Dietary samples were collected in lowland tropical forest of central Peninsular Malaysia. A general invertebrate primer pair was used for the first time to assess diet of tropical birds. The USEARCH was used to cluster the COI mtDNA sequences into Operational Taxonomic Unit (OTU). OTU sequences were aligned and queried through the GenBank or Biodiversity of Life Database (BOLD). We identified 26 distinct arthropod taxa from 31 OTUs. Of all OTUs, there was three that could be identified up to species level, 20 to genus level, three to family level and five could not assigned to any taxa (the $_{BLAST}$ hits were poor). All sequences were identified to class Insecta belonging to 18 families from four orders, where Lepidoptera representing major insect order consumed by study bird species. This non-invasive molecular approach provides a practical and rapid technique to understand of how energy flows across ecosystems. This technique could be very useful to screen for possible particular pest insects consumed by insectivores (e.g. birds and bats) in crop plantation. A comprehensive arthropod studies and local reference sequences need to be added to the database to improve the proportion of sequences that can be identified.*

Keywords: Dietary ecology; MiSeq; next-generation sequencing (NGS); Philentoma pyrhoptera*; tropical birds*

ABSTRAK

*Kajian diet memberi pemahaman tentang hubungan antara pemangsa-mangsa, namun diet burung di hutan tropika kurang difahami. Dalam kajian ini, satu kaedah yang tidak invasif, penjujukan generasi akan datang (platform Illumina MiSeq) digunakan untuk mengenal pasti mangsa dalam sampel najis Filentoma Sayap Merah (*Philentoma pyrhoptera*). Sampel makanan diambil di hutan tropika tanah pamah di Semenanjung Malaysia. Set primer umum untuk invertebrata digunakan pertama kalinya untuk menilai diet burung tropika. Pautan USEARCH digunakan untuk mengkelompok jujukan mtDNA COI kepada Unit Operasi Taksonomi (OTU). Jujukan OTU telah disunting menggunakan perisian BioEdit dan ditentukan menerusi Pangkalan Data GenBank atau Biodiversity of Life Database (BOLD). Kami mengenal pasti 26 taksonomi arthropoda yang unik daripada 31 OTUs. Daripada semua OTUs, terdapat tiga yang boleh dikenal pasti hingga ke tahap spesies, 20 hingga genus, tiga hingga famili dan lima tidak dapat ditaksirkan (kadar $_{BLAST}$ yang rendah). Semua jujukan dikenal pasti sebagai kelas Insecta yang terdiri daripada 18 famili daripada empat order dengan Lepidoptera mewakili order serangga yang utama dimakan oleh spesies burung kajian. Pendekatan molekul yang tidak invasif ini menyediakan teknik yang praktikal dan cepat untuk memahami bagaimana tenaga mengalir merentasi ekosistem. Teknik ini juga sangat berguna untuk melihat kemungkinan serangga perosak yang tertentu dimakan oleh insektivor (contohnya, burung dan kelawar) di ladang tanaman. Kajian artropoda yang komprehensif dan jujukan rujukan tempatan perlu ditambah ke pangkalan data untuk meningkatkan peratusan jujukan yang boleh dikenal pasti.*

Kata kunci: Burung tropika; ekologi pemakanan; MiSeq; penjujukan generasi akan datang (NGS); Philentoma pyrhoptera

INTRODUCTION

Assessing predator-prey relationships provide insights into many ecological aspects of particular species (Naoki 2007). Information on food sources that sustain many bird species in particular habitat is important to protect threatened species (Li et al. 2014) and to understand the role of birds in forest trophic webs (Mäntylä et al. 2011). Dietary studies provide insights in predator-prey relationships that defined the energy flows across ecosystems. It is vital to show the dietary ecology of an animal as it is a basic element in constructing their niche, thus maintaining terrestrial ecosystems at the population and community levels (Baxter et al. 2005). For instance, insectivorous birds play an important role to regulate the trophic flows (Schmitz et al. 2010) and serve as pest biocontrol (King et al. 2015) as well as bio-indicators in evaluating habitat disturbance (Mansor & Sah 2012; Yong et al. 2011).

Optimal dietary theory suggests that dietary specialization is reflected by prey availability, abundance, diversity and quality (MacArthur & Pianka 1966). Optimal foraging theory also predicts that predators are selective when faced with abundant prey, but become less picky when prey gets sparse (Emlen 1966). However, foraging opportunism may also occur in certain bird species, leading to dietary generalization and allowing niche separation through wider diet preferences (Sherry et al. 2016), which were useful when food sources are limited (Terraube et al. 2011).

In recent years, molecular technique have provided far more detailed dietary analyses than morphological studies by providing lower taxonomic level, ranging from family to species (Pompanon et al. 2012). Molecular diagnostic provides a non-invasive tool to obtain dietary information. It is proved that the DNA of prey can still be detected in faecal samples of birds for period of time (King et al. 2008; Wong et al. 2015). Remaining degraded DNA in diet of predators can be amplified using short base-pair universal primers (King et al. 2008), followed by cloning and Sanger sequencing (Jedlicka et al. 2013), or by recent Next-generation Sequencing (NGS) through Illumina MiSeq platform. The latter technique provides a rapid dietary screening of numerous prey taxa that present in predator faecal in a single run (Sint et al. 2012).

It is known that Rufous-winged Philentoma feed on insects and other arthropods (Wells 2007), but detail taxonomic level data (i.e. order, family, genus) of their diet is incomplete. Due to their aerial snatching for insect, it is difficult to observe and detect their prey types in the field. Moreover, rapid digestion rate in birds are likely reduced possibility to identify prey from dietary samples to lower taxonomical identification (i.e., family or genus) using morphological technique. Therefore, most previous dietary studies of birds were rarely providing identification beyond the order level. Rufous-winged Philentoma is listed as totally protected animal in Malaysia Wildlife Conservation Act 2010 (Wildlife Act 2010), thus applying such non-invasive methods to analyze dietary samples are crucial and needed for conservation and management plans.

Here we used advance molecular NGS technique to describe the diet of Rufous-winged Philentoma (*Philentoma pyrhoptera*) using the Illumina MiSeq. Specifically, this study addressed the following questions: Do current molecular analysis technique suitable for dietary study of tropical forest birds?; and What prey types (i.e., species, genus and family) does the insectivorous bird consume in lowland tropical forest? This study present for the first time molecular dietary information of Malaysian forest bird, subsequently provide better understanding of diet ranges in tropical birds.

Materials and Methods

Study Area

The study was conducted in Bukit Rengit, within the Krau Wildlife Reserve, Pahang, central Peninsular Malaysia (Figure 1). This reserve is approximately 610 km^2 in size and a second largest protected area in Peninsular Malaysia after Taman Negara. The elevation ranges from 50 m at Kuala Lompat to over 2000 m at the summit of Mount Benom. The region is mainly comprised of mature dipterocarp forest, with large area of old-growth forest (Clark 1996). The annual daily mean temperature of the

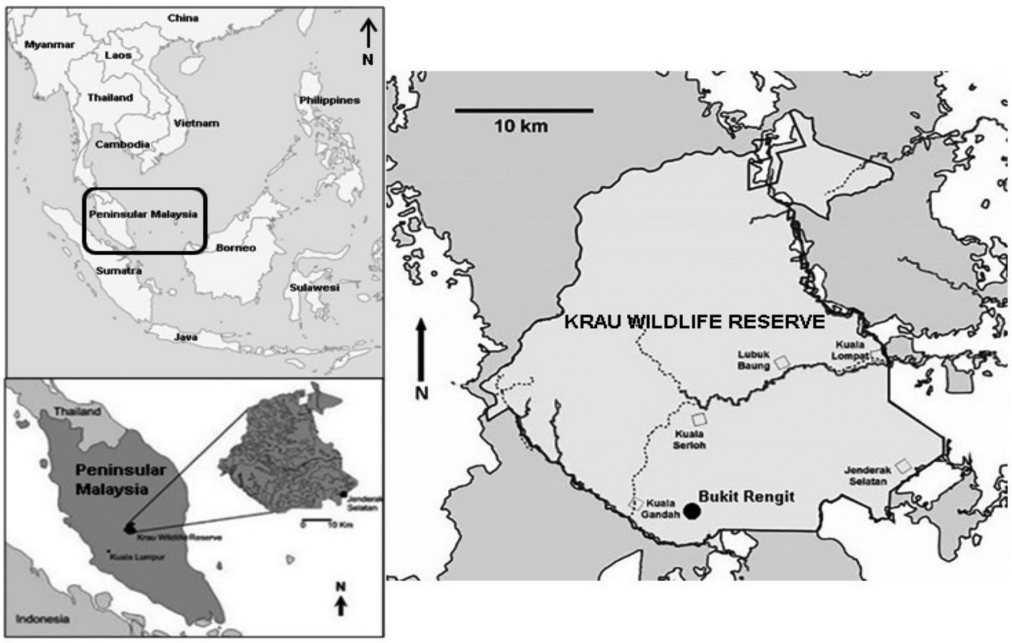

FIGURE 1. Location of study area; the Krau Wildlife Reserve, located in Pahang, Peninsular Malaysia. Light grey denoted reserve area, dark grey represents forested areas surrounding the reserve, while white indicates a non-forested area

area is about 26.4ºC and average rainfall is in the range of 2,000-2,200 mm throughout the year and separated with two drier periods (< 100 mm/month), between January to April and June to August (Chua & Saw 2006).

SAMPLE COLLECTION

Data were collected between February 2014 and September 2015. A total of 15 mist nets (12 m, 30 mm mesh) were positioned at various locations along three forest trails. All captured birds were identified, weighed, measured and banded with metal rings. Selected insectivorous bird species were separately placed in clean cotton bags and were released as soon as faecal samples were collected. Faeces were also collected opportunistically from net-disentangling process during bird-ringing sessions. These samples were immediately preserved in 99.8% absolute ethanol and later stored under -20ºC.

DNA EXTRACTION, PCR AMPLIFICATION AND SEQUENCING

DNA was extracted from faecal samples using the NucleoSpin® Soil Kit (Macherey-Nagel GmbH & Co., Germany). All extraction steps were performed according to the manufacturer's protocol, with some modifications. A 286 base-pair length fragment of the mitochondrial DNA cytochrome c oxidase subunit I barcode region (COI) was amplified from each DNA extract using the forward primer LCO1490 (5'-GTCAACAAATCATAAAGATATTGG-3') and the reverse primer HCO1777 (5'-ACTTATATTGTTTATACGAGGGAA-3') (Brown et al. 2012). Amplifications were performed in triplicate to validate the results, with 20 µL PCR mixture containing 4 µL of 5 × FastPfu Buffer, 2 µL of 2.5 mM dNTPs, 0.8 µL of each primer (5 µM), 0.4 µL of FastPfu Polymerase TransGen Biotech, China) and 10 ng of template DNA. PCR was done using Applied Biosystems Veriti 96-Well Thermal Cycler (Applied Biosystems, Inc. USA). After an initial denaturing step at 94ºC for 2 min 30 s, amplification proceeded for 35 cycles at 94ºC for 30 s, 44ºC for 30 s, 72ºC for 45 s and a final extension at 72ºC for 10 min. The PCR amplicons were extracted from 2% agarose gels and purified using the AxyPrep DNA Gel Extraction Kit (Axygen Biosciences, USA.), following the manufacturer's protocols and quantified using QuantiFluor™ -ST (Promega, USA). The primer set was modified by the addition of Illumina primer sequence and Illumina's Nextera overhang adaptors on the 5' ends. Sample libraries were normalized, pooled in equimolar and sequenced (2 × 250/300 bp) on the MiSeq Desktop Sequencer (Illumina, USA), following the standard protocols.

DATA ANALYSIS

Amplicon sequences obtained from the Illumina MiSeq were filtered and collapsed into unique haplotypes (singleton removed) and the clustered into Operational Taxonomic Unit (OTU) using the USEARCH v8.1.1861 (Edgar 2013) default, 97% similarity threshold OTU sequences were aligned and queried through the GenBank (http://www.ncbi.nlm.nih.gov/) or Biodiversity of Life Database (BOLD) (http:// www.boldsystems.org/; Ratnasingham & Hebert 2007). A sequence that has at least 98% similarity to any other reference sequence was identified to the species level. The sequence was identified to higher taxonomic level when sequence could not be clearly matched to a single species, possibly due to the incomplete database to family when similarity exceeded 90%, and to genus when similarity exceeded 95%.

RESULTS

We recovered 52,813 sequencing reads from dietary samples of Rufous-winged Philentoma. After bioinformatics processing, we reduced these reads to 16,901 unique haplotypes, which were then clustered into 31 OTUs. All blast hits were assigned to class Insecta belong to 18 families from four orders. Of all OTUs, we identified 26 distinct arthropod taxa (Table 1), where the similarity to reference database ranged from 94% to 100%. Three OTU sequences could be identified up to species level (≥ 98%-100% similarity), 20 to genus level (> 95%-100% similarity), three to family level (> 90%-100% similarity) and five could not assigned to any taxa (the $_{BLAST}$ hits were poor). Three insect species identified were the *Orgyia araea* (Lepidoptera), *Telicota colon* (Lepidoptera), and *Odontomachus simillimus* (Hymenoptera). Lepidopterans represent major insect group consumed by study bird species, followed by coleopterans, hymenopterans and dipterans (Figure 2). Erebidae from order Lepidoptera showed the highest insect family presence in diet of Philentoma (Figure 3).

DISCUSSION

Analyses showed that Lepidoptera was the most dominant food source for the Rufous-winged Philentoma. This result corresponded with their sallying ability to catch flying insect (e.g. butterflies) and herbivorous insect (e.g. caterpillars). Rufous-winged Philentoma is known to flycatch the insect from both air and live green leaves (Mansor & Ramli 2017). Preferences on lepidopterans was also documented in dietary studies of other aerial insectivores (i.e. bats) using NGS platforms (Clare et al. 2014; Salinas-Ramos et al. 2015), but rarely in diet of birds. Dietary analyses such as this can tell where birds or other animal groups are most likely to forage and what they have eaten.

Diet variability in Philentoma is expected corresponded to microclimatic conditions that change seasonally (Razgour et al. 2011). It is thought that the insectivores' diet may responds to arthropod population fluctuations (Clare et al. 2011). Its fluctuations possibly associated with the amount of rainfall (Borghesio & Laiolo 2004). Insectivores' diets were found to be more generalize in the dry season when insect abundance become more limited

TABLE 1. Similarity of OTU insect sequences obtained from Rufous-winged Philentoma faecal to either GenBank or BOLD reference databases

Order	Family	Species	Similarity (%)
Coleoptera	Carabidae	Carabidae sp.	94.02
	Curculionidae	Curculionidae sp.	98.43
	Elateridae	Elateridae sp.	98.36
		Abelater sp.	95.24
	Staphylinidae	*Aleochara* sp.	95.45
Diptera	Dixidae	*Dixella* sp.	95.89
	Syrphidae	*Sericomyia* sp.	95.56
Hymenoptera	Formicidae	*Odontomachus simillimus*	98.81
		Pachycondyla sp.	98.81
Lepidoptera	Blastobasidae	*Blastobasis* sp.	95.5
	Depressariidae	*Depressaria* sp.	99.1
	Erebidae	*Catocala* sp.	99.12
		Eubryopterella sp.	97.65
		Hypocala sp.	97.18
		Orgyia araea	99.52
	Hesperiidae	*Telicota colon*	100
		Pyrgus sp.	99.07
	Lycaenidae	*Euphilotes* sp.	98.96
	Noctuidae	*Mythimna* sp.	97.53
	Notodontidae	*Elasmia* sp.	100
	Nymphalidae	*Hamadryas* sp.	97.28
		Junonia sp.	96.55
	Oecophoridae	*Artiastis* sp.	99.16
		Eulechria sp.	100
	Saturniidae	*Loxolomia* sp.	100
	Xyloryctidae	*Cryptophasa* sp.	98.89

(Razo-Gonzalez et al. 2014), probably to cope with the reduction of certain arthropod groups. Optimal foraging theory predicts that predators are selective (specialized) when faced with abundant prey, but become less picky (generalized) when prey gets sparse (Emlen 1966). The detection of broad prey taxa in present study could act as important tool to describe the food web structure in communities (Clare et al. 2009).

Although sample size were low (n = 9), this study provide useful information that give a complete picture of diet of tropical insectivorous birds. Overall, the Rufous-winged Philentoma exhibited fairly diverse diet, indicating sample size may not really important in molecular diet analysis because the tendency of NGS and a correct PCR primer sets to help in amplification of numerous arthropod sequences. To ensure successful amplification of prey DNA, we used PCR primer set targeting small DNA fragment (<300 bp), as large DNA fragments are digested relatively quickly in bird's digestive tract (King et al. 2015). We proved that this primer set was specific to invertebrates, although some of the sequences could not be assigned to any taxa. We recommend using this primer set in future diet studies of all insectivores in tropical regions.

The results showed that reference database, GenBank (NCBI) and BOLD-IDS, have ability to provide up to species, genus, or family level identification of arthropods in diet of tropical forest birds. However, several OTU sequences could not be assigned to any taxa (the $_{BLAST}$ hits were poor). Molecular approaches may be limited in certain regions, especially in the tropics where reference database are much less barcoded (King et al. 2015). Therefore, a comprehensive arthropod studies and local reference sequences need to be added to the database to improve the percentage of sequences that can be identified. Dietary studies in well-barcoded regions such as Europe (Clare et al. 2014; Hope et al. 2014; King et al. 2015; Vesterinen et al. 2016) is much more successful with numerous sequences matched to species. Nonetheless, present study provides novel findings on arthropods consumed by tropical bird at lower taxonomic level, mostly genus level. This study also revealed that NGS can provide a quick dietary screening that provide early possible information on diet of all animal groups, and offer strategies in biodiversity and conservation program.

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