Current Zoology, 2022, **68**, 381–389
https://doi.org/10.1093/cz/zoab074
Advance access publication 3 September 2021
Original Article

High-throughput sequencing reveals dietary segregation in Malaysian babblers

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Handling Editor: David Swanson

Abstract

The coexistence of numerous species within a community results from how those species use available resources. Babblers are one of the major groups of Malaysian insectivorous birds, which frequently forage in dense vegetation cover and have a high level of sympatry. Therefore, examining the diet, prey selection, and niche segregation of babblers can be challenging. In this study, we used high-throughput sequencing to investigate potential dietary overlap or segregation among 10 babbler species of the 4 genera of the family Pellorneidae and Timaliidae: *Pellorneum*, *Malacopteron*, *Stachyris*, and *Cyanoderma* in central peninsular Malaysia. We tested the hypothesis that trophically similar species may differ in resource use to avoid competitive exclusion. We identified 81 distinct arthropod taxa from fecal samples, belonging to 71 families representing 13 orders, which were predominantly from 16 dipteran, 13 lepidopteran, and 10 coleopteran families. Of all the prey taxa consumed, 45% were found to be distinct across the 10 babbler species, and <35% were shared simultaneously by ≥3 babbler species, indicating minimal dietary overlap. The black-throated babbler *Stachyris nigricollis* and moustached babbler *Malacopteron magnirostre* had the most generalist tendencies because they consumed a greater variety of prey taxa. Small dietary overlap values (Ojk) and a relatively wide range of food resources suggest that dietary segregation occurred among the studied babblers. The great diversity of prey consumed revealed the presence of dietary flexibility among the sympatric insectivorous birds, thus reducing any active dietary competition and facilitating the coexistence through niche partitioning.

Keywords: coexistence, dietary partitioning, metabarcoding, next-generation sequencing, tropical insectivorous birds

Babblers are a major component of the tropical Asian avifauna. Most babblers have a high level of sympatry, are confined to the forest interior and forage predominantly on aerial leaf litter in the understory (Mansor and Ramli 2017). Babblers are among the predominant Malaysian insectivorous birds (Yong et al. 2011) and are important for the regulation of forest ecosystem services. Insectivorous birds play an important role in regulating trophic flows (Schmitz et al. 2010) as well as reducing the levels of pest and herbivorous insects (Karp and Daily 2014; King et al. 2015) and forest defoliation (Eveleigh et al. 2007). Babblers are believed to typically feed on a variable range of arthropod taxa (Wells 2007), however some prey selectivity has been reported (Mansor et al. 2018). Because of their small size and habits of foraging in dense vegetation cover, it can be challenging to observe and detect their prey selection in the field. Moreover, because of the rapid digestion rate in birds, the possibility of identifying prey from dietary samples to a lower taxonomic level through gut content and regurgitation sample analyses is limited. Therefore, most previous dietary studies have rarely identified prey beyond the order level (e.g., Manhães et al. 2010; Sherry et al. 2016; Mansor et al. 2018). Such traditional morphological diet assessments suffer from overrepresentation of hard-bodied prey, underrepresentation of soft-bodied prey, and misidentification of very small and morphologically cryptic arthropods (Pompanon et al. 2012; Kress et al. 2015).

In recent years, molecular techniques have provided more effective dietary analyses than traditional morphological and behavioral studies and have allowed identification to lower taxonomic levels, ranging from family to species (Mata et al. 2019). Molecular scatology makes it possible to analyze the arthropod prey of avian predators (McClenaghan et al. 2019). It has been proven that the DNA of prey can still be detected in fecal samples of birds for a period of time (King et al. 2015). The remaining degraded DNA from the diets of predators can be amplified using short base pair (bp) universal primers, followed by cloning and Sanger sequencing (Jedlicka et al. 2013) or, more recently, by high-throughput sequencing (HTS) through the Illumina MiSeq platform. Despite the limitations of HTS approach, particularly the lack of biomass quantification, many of the dietary studies conducted in the last 5 years have employed similar methods.

Received 6 April 2021; revised 27 August 2021; accepted 1 September 2021

The advancement of molecular HTS in providing the ability to effectively identify prey consumed at lowest taxonomic resolution is making it possible to overcome the limitations of these techniques (Nielsen et al. 2018). HTS approach provides rapid dietary screening of the numerous prey taxa present in a single sample and can act as a noninvasive tool to obtain dietary information of a wide range of animals (Kolkert et al. 2020; Hacker et al. 2021; Johnson et al. 2021; Pertoldi et al. 2021), including birds (Sullins et al. 2018; Chan et al. 2019; da Silva et al. 2020; Evens et al. 2020; Mansor et al. 2020a). As the technology has spread and costs reduced, ecologists have rapidly turned to this powerful, novel tool for ecological studies, including dietary analysis. However, applying HTS to simultaneously evaluate diets of sympatric species remains under evaluated (Trevelline et al. 2018), particularly small passerine forest birds in tropical region.

Assessing predator–prey relationships at the finest taxonomic level using molecular HTS techniques can help to define the energy flows across ecosystems and provide insights into many ecological aspects, such as predator behavior and population dynamics. This offers an opportunity to assess diet preferences and possible resource segregation of several bird species inhabiting the same habitat. Understanding the coexistence of sympatric species within communities depends on elucidating their use of varying resources (Goodyear and Pianka 2011). Hardin (1960) hypothesized that trophically similar species may differ in resource use to avoid competitive exclusion, which allows them to coexist. Morphological adaptation and foraging specialization allow species to coexist in the same habitat through niche partitioning (Mansor and Ramli 2017). In addition to dietary segregation through selective feeding and specialization, a broad and flexible diet can also occur in birds (Orłowski and Karg 2013; McClenaghan et al. 2019), suggesting that the consumption of a wider variety of prey can help reduce dietary competition. However, limited dietary niche overlap among closely related species in the same habitat may occur, especially when resources are restricted (Crisol-Martínez et al. 2016; Sherry et al. 2016). Thus, understanding the degree of overlap in the utilization of food resources among sympatric species is crucial.

In this study, we apply the HTS approach to examine the diets of 10 babbler species: the black-capped babbler (BCB) *Pellorneum capistratum*, white-chested babbler (WCB) *Pellorneum rostratum*, ferruginous babbler (FB) *Pellorneum bicolor*, scaly-crowned babbler (SCB) *Malacopteron cinereum*, rufous-crowned babbler (RCB) *Malacopteron magnum*, moustached babbler (MB) *Malacopteron magnirostre*, gray-headed babbler (GHB) *Stachyris poliocephala*, black-throated babbler (BTB) *Stachyris nigricollis*, chestnut-rumped babbler (CRB) *Stachyris maculata*, and chestnut-winged babbler (CWB) *Cyanoderma erythropterum*. Specifically, this study addressed the following questions: (1) What are the main prey types (i.e., species, genus, family, and order) consumed by the key Malaysian insectivorous birds in lowland tropical forests? (2) Do babblers partition food resources or use overlapping resources to stably coexist in the same habitat? Molecular-based dietary studies have rarely concentrated on multiple species of predators simultaneously, particularly birds, in the same geographical area. Therefore, the findings of this study will reveal the dietary breadth and overlap of multiple closely related forest insectivorous bird species within the same habitat of lowland tropical rainforests, and allow an understanding of the ecological principles of niche partitioning. All of the studied babblers are categorized as "totally protected" wildlife in the Malaysian Wildlife Conservation Act 2010 (Wildlife Act 2010), and 4 species have been listed as "near-threatened" by the International Union for Conservation of Nature's Red List of Threatened Species (IUCN 2020); thus, applying HTS methods to analyze dietary samples is crucial and needed for conservation and management plans. We demonstrate the strength of HTS to resolve trophic ecology questions and to reveal the unseen foraging patterns of small forest passerine birds.

Materials and Methods

Study area

The study was conducted in Bukit Rengit (3°35-52′ N, 102°05-17′ E), in the southern part of the Krau Wildlife Reserve, a protected area located in Pahang, central peninsular Malaysia (Figure 1). This reserve is the largest wildlife reserve in peninsular Malaysia, with an approximate size of 62,000 ha. The elevation of the reserve ranges from ~50 m at Kuala Lompat to over 2,100 m at the summit of Mount Benom and is drained by 3 major river systems: the Sungai Krau, Sungai Lompat, and Sungai Teris. The Krau Wildlife Reserve is mainly comprised of mature dipterocarp forest, with a large area of old-growth forest (Clark 1996). It is dominated by lowland, hill and upper dipterocarps, riverine, montane oak-laurel, and montane forest (DWNP/DANCED 2001). The associated dominant tree species in the reserve include *Anisophyllea corneri*, *Mallotus penangensis*, *Gymnacranthera forbesii*, *Shorea macroptera*, *Shorea maxwelliana*, *Shorea lepidota*, and *Elateriospermum tapos* (Nizam et al. 2006).

The daily temperature varies between a minimum of 23 °C and a maximum of 33 °C, and maximum rainfall occurring between October and January, average ~1,500 mm. All 5 research stations in this reserve; Kuala Lompat Research Station, Lubuk Baung, Kuala Sungai Serloh, Kuala Gandah, and Jenderak Selatan, are managed by the Department of Wildlife and National Parks Peninsular Malaysia.

Sample collection

A total of 16 mist nets (12-m, 30-mm mesh) were positioned near the ground at various locations along 3 forest trails for 12 days every month, during the wet season (from October 2014 to January 2015). The length of the trails varied from 500 to 800 m and each trail was separated from the others by ~150 m. At each site, 1 net was set parallel and the other perpendicular to the trail. The nets were deployed simultaneously from 0800 to 1,500 h, to allow captured birds to forage before and after sample collection in order to avoid empty stomachs. After being caught, birds were placed into sterile cotton bags. Due to the high sensitivity to disturbance and shy foraging habits of babbler, it can be challenging to get larger sample size. We acknowledged this limitation by sampled at least 10 individuals for each babbler species, following Emrich et al.'s (2014) dietary study of several sympatric species.

Once a fecal sample was produced, the bird was identified, weighed, measured, and banded with metal rings to record repeated sampling, and released. The fecal samples were collected from the bags within 10 min of defecation using sterilized forceps. Fecal samples were also collected opportunistically during the net-disentangling process. After collection, all fecal samples were immediately stored in 99.8% ethanol

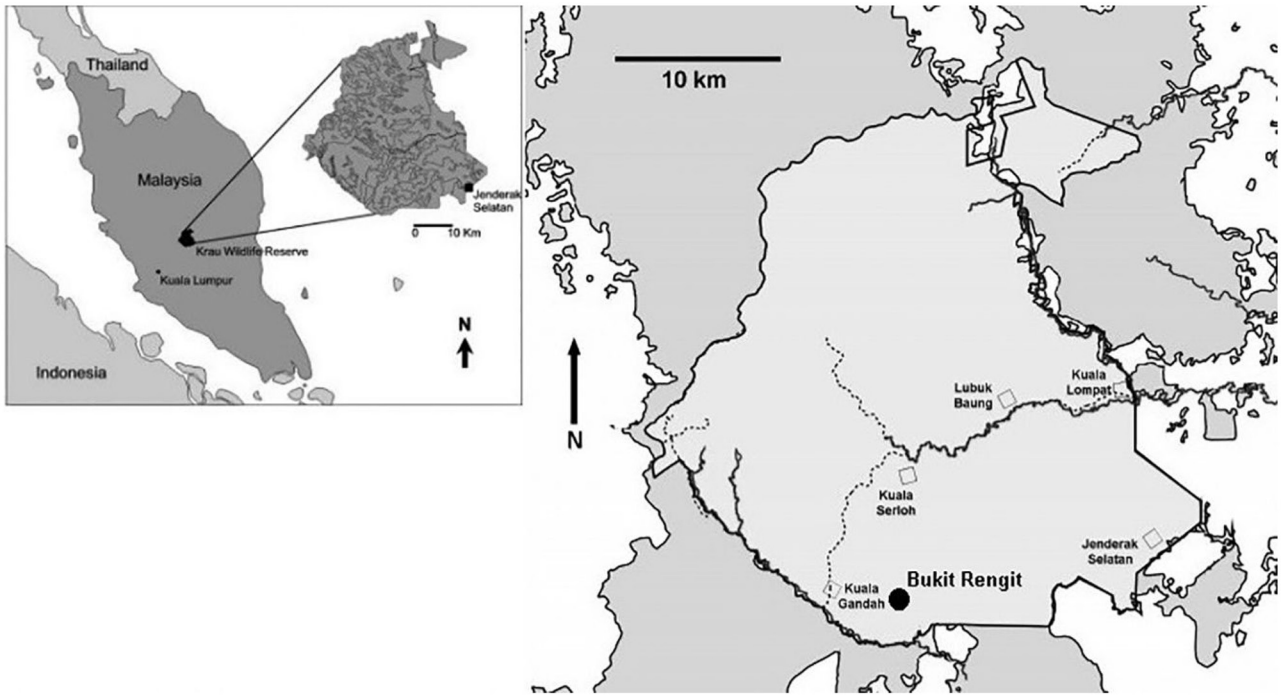

Figure 1. Map of Krau Wildlife Reserve, Pahang, Peninsular Malaysia. Light gray denotes the reserve area, dark gray represents the forested areas surrounding the reserve, whereas white indicates non-forested areas. Map adapted from Zakaria et al. (2014).

and stored upon return from the field in a –20 °C freezer, and later transferred to a –40 °C freezer, where they were stored until DNA extraction.

DNA extraction, PCR amplification, and sequencing

The genomic DNA was extracted from the fecal samples using the NucleoSpin® Soil Kit (Macherey-Nagel GmbH & Co., Germany) according to the manufacturer's protocol. A 286-bp target region of cytochrome c oxidase I was amplified using the LCO1490 (Folmer et al. 1994) and HCO1777 (Brown et al. 2012) primer set. The amplifications were performed in triplicate in a 20 µL mixture containing 4 µL of 5 × FastPfu Buffer, 2 µL of 2.5 mM dNTPs, 0.8 µL of 5 µM each of the forward and reverse primers, 0.4 µL of FastPfu Polymerase, and 10 ng of template DNA. Thermocycling conditions were as follows: 2 min 30 s at 94 °C; followed by 35 cycles of 30 s at 94 °C, 30 s at 44°C and 45 s at 72 °C; followed by a final extension at 72 °C for 10 min. Amplicons were extracted from 2% agarose gels and purified using an AxyPrep DNA Gel Extraction Kit (Axygen Biosciences, San Francisco, CA) according to the manufacturer's protocols. The cleaned products were quantified using QuantiFluor-ST (Promega, Madison, WI). The sample libraries were pooled in equimolar concentrations and paired-end sequenced (2 × 300 bp) on an Illumina MiSeq platform (San Diego, CA).

Data analysis

The amplicon sequences obtained from the Illumina MiSeq were quality-filtered, full-length duplicate sequences were removed, and collapsed into unique haplotypes (singletons were removed) using USEARCH version 9 (Edgar 2010). The trimmed, quality-filtered sequences were clustered into prey Operational Taxonomic Units (OTUs) with a specified 97% sequence similarity threshold. The OTU sequences were queried through GenBank (http://www.ncbi.nlm.nih.gov/) and the Biodiversity of Life Database (BOLD) (http://www.boldsystems.org/, Ratnasingham and Hebert 2007). Species-, genus-, and family-level identifications were assigned at a minimum of 97, 95, and 90% similarity, respectively, based on Zeale et al. (2010) and Mansor et al. (2020a). Sequences identified as the host (i.e., birds) or non-arthropods (e.g., algae or nematodes) and sequences that were not resolved to at least the family level, were omitted from the analysis.

We quantified the diet composition of study babblers using the frequency of occurrence, by dividing number of samples in which an order was detected with the number of all the samples, and the relative read abundance of prey orders. The occurrence of prey taxa (e.g., different arthropod families) across samples can be used as a semiquantitative measure to compare how different prey taxa are consumed by predators (Bowser et al. 2013). One-way analysis of variance (ANOVA) was used to examine differences in diet among study babbler species as expressed as the percentage of prey taxa (Sokal and Rohlf 1995; Krüger et al. 2012), and the data were arcsine-transformed before analysis. Pairwise similarity indices were calculated using Pianka's (1973) measure of niche overlap (Pianka's index, Ojk) to assess dietary overlap among the 10 babbler species, in which a value near 0 indicates no common use of dimensions, whereas a value of 1 indicates complete dietary overlap. These analyses were performed using ECOSIM version 7.71 (Gotelli and Entsminger 2001). To assess the dietary breadth of the studied babbler species, Shannon diversity (H′), in which higher H′ values indicate greater species diversity, was computed using the Paleontological Statistics Software (PAST) package (Hammer et al. 2001). A Spearman rank correlation and a hierarchical cluster analysis using the Bray–Curtis index were also performed in the PAST package to assess variation in prey taxa across 10 babbler species and to group the species into distinctive feeding guilds.

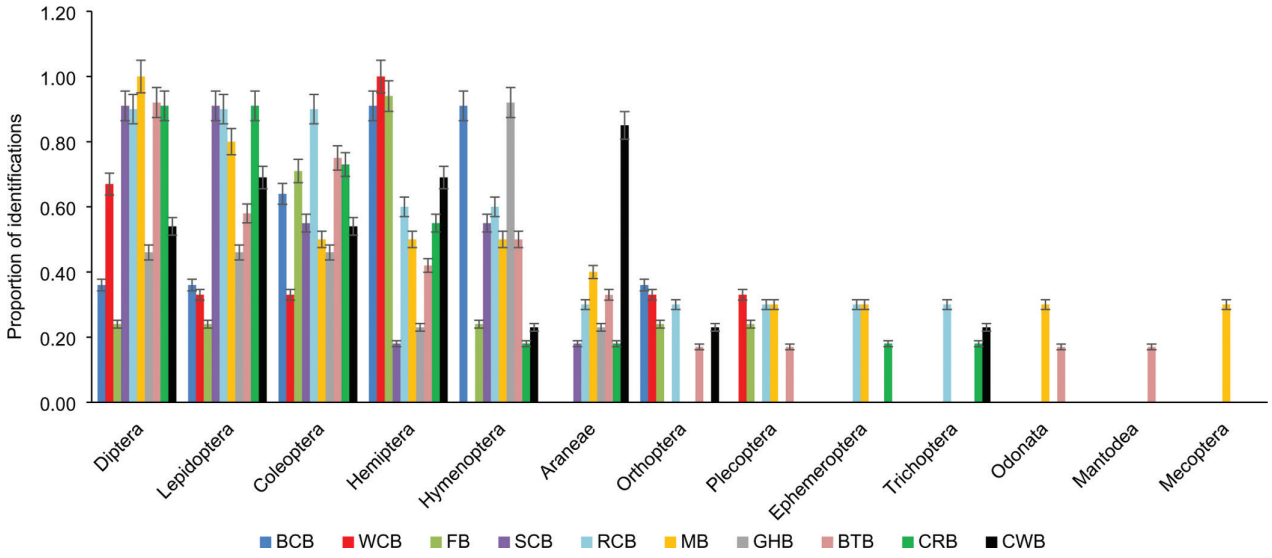

Figure 2. The proportions FOO of prey in feces of the 10 babbler species. Each bar on the *x*-axis represents a different prey taxon, and the bars are colored by babbler species. The 5 most frequently consumed arthropod taxa were from the orders Diptera, Lepidoptera, Coleoptera, Hemiptera, and Hymenoptera.

Results

Prey identity

We recovered 465,916 sequencing reads from the fecal samples of the 10 babbler species. After bioinformatics processing, these reads were quality-filtered to 140,346 unique haplotypes, which were then clustered into 194 OTUs. Altogether, 81 distinct arthropod taxa were identified and most of the blast hits were assigned to the class Insecta (74) and a small proportion was assigned to Arachnida (7), with similarity to the reference databases ranging from 90% to 100% (Supplementary Table S1). Among these classes, 13 orders and 71 families were represented, which were predominantly from dipteran (16), lepidopteran (13), and coleopteran (10) families (Figure 2; Supplementary Table S2). Five of the genera belonged to the Hymenoptera and Diptera, 2 to the Coleoptera, and 1 to the Araneae. The arthropod species detected were *Xyleborus volvulus*, belonging to the Coleoptera, *Monomorium pharaonis*, and *Odontomachus simillimus*, belonging to the Hymenoptera, and *Pantala flavescens*, belonging to the Odonata. On average across all 10 babbler species, 21% of the occurrences were from the Diptera, 18% from the Lepidoptera, 17% from the Coleoptera, and 15% from the Hemiptera.

Prey selection

BTBs had the most generalist tendencies, consuming the widest range of arthropod taxa (n = 40, H′ = 3.638), followed by MBs (n = 29, H′ = 3.332), CWBs (n = 22, H′ = 3.091) and CRBs (n = 21, H′ = 2.996) (Table 1). BTBs predominantly fed on dipterans and coleopterans; MBs fed on dipterans and lepidopterans; CWBs fed on arachnids and hemipterans; and CRBs fed on dipterans and lepidopterans (Figure 3; Supplementary Table S3). WCBs, which mainly fed on hemipterans, were the most specialized, having the lowest diversity (n = 9, H′ = 2.197) for the arthropod taxa they consumed.

Table 1. Shannon diversity index of the prey identified from 10 babbler species

Bird species	Shannon (H′)	*N*
BCB	2.398	11
WCB	2.197	12
FB	2.485	17
SCB	2.890	11
RCB	2.996	10
MB	3.332	10
GHB	2.398	13
BTB	3.638	12
CRB	2.996	11
CWB	3.091	13

Dietary segregation

Overall, we found low levels of dietary overlap among the 10 babbler species examined (ANOVA: $F = 6.239$, $P < 0.01$), with a mean degree of overlap <0.4 Ojk. Most values ranging from 0.20 to 0.40 (Table 2) except for the degree of overlap between WCBs and FBs (Ojk = 0.674, Table 2). Of all the prey taxa consumed, 38 were found to be distinct across study babbler species (Supplementary TableS2). The remaining 43 prey taxa were rarely shared by ≥3 babbler species. The Tortricidae family from the order Lepidoptera, which was present in the diets of 9 babbler species, showed the highest overlap, followed by the Pentatomidae and Miridae families from the order Hemiptera (8 and 7 babbler species, respectively). The other 2 major families, Oecophoridae from the order Lepidoptera, and Cicadidae from the order Hemiptera, were present in the diets of 6 babbler species. *Pachycondyla* sp., which was present in the diets of 6 babbler species, was the insect genus with the highest rate of overlap. *Odontomachus simillimus* and *X. volvulus*, which were present in the diets of 6 and 4 babbler species, respectively,

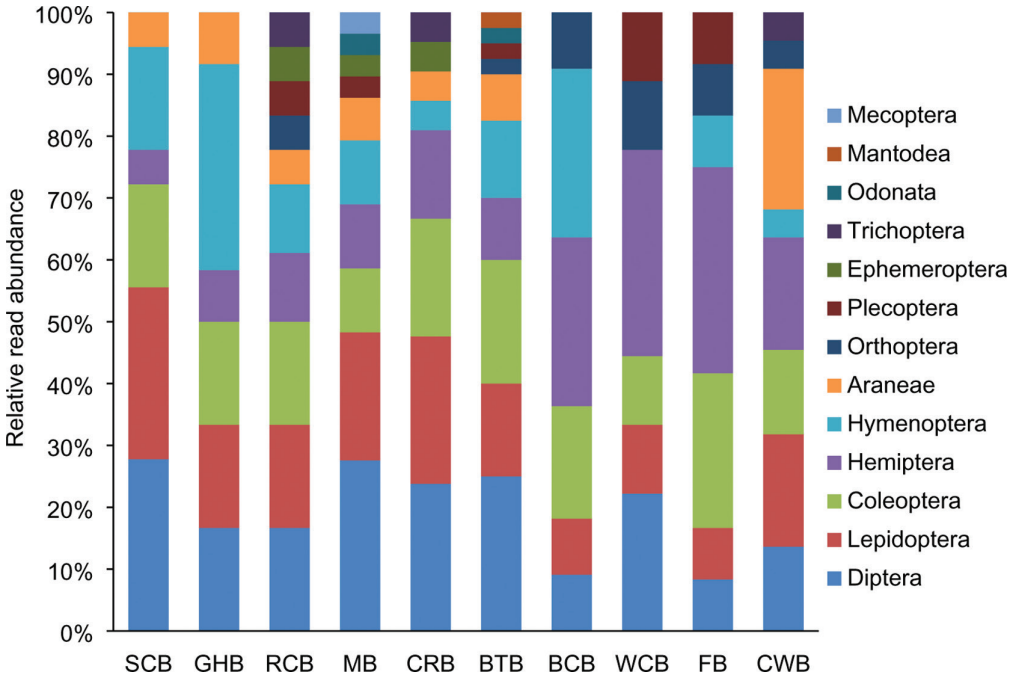

Figure 3. RRA of prey items at the order level. Diptera, Lepidoptera, and Coleoptera accounted a large proportion of the total OTUs.

Table 2 Pairwise similarity indices using Pianka's measure of dietary overlap (Ojk) across the 10 babbler species

Babbler species	WCB	FB	SCB	RCB	MB	GHB	BTB	CRB	CWB
BCB	0.201	0.435	0.284	0.284	0.336	0.261	0.429	0.197	0.193
WCB	—	0.674	0.157	0.314	0.248	0.096	0.264	0.218	0.500
FB	—	—	0.204	0.340	0.322	0.250	0.274	0.315	0.431
SCB	—	—	—	0.333	0.394	0.340	0.335	0.309	0.352
RCB	—	—	—	—	0.438	0.272	0.224	0.360	0.352
MB	—	—	—	—		0.429	0.352	0.486	0.317
GHB	—	—	—	—	—	—	0.365	0.252	0.123
BTB	—	—	—	—	—	—	—	0.380	0.303
CRB	—	—	—	—	—	—	—	—	0.279

were among the arthropod species with the highest rates of overlap.

Dietary segregation among the babbler species is clearly demonstrated by hierarchical cluster analysis (Figure 4). The analysis divided the 10 babbler species into 4 main feeding guilds based on the consumption of prey taxa. Feeding Guild 1 included the SCB and GHB; feeding Guild 2 included RCB, MB, and CRB; feeding Guild 3 included the BTB and BCB; and feeding Guild 4 included the WCB, FB, and CWB. The WCB and FB have the highest dietary similarity index (Table 3).

Discussion

We demonstrated that the diets of the 10 sympatric babbler species studied varied significantly and overlapped slightly. Of all the prey taxa consumed (mostly at the family level), 45% were found to be distinct among the 10 babbler species, and <35% were shared by 3 or more babbler species (Supplementary Table S2), indicating minimal dietary overlap. This variation in diet suggests the existence of dietary segregation, which reduces interspecific competition and may be interpreted based on the tendencies of birds to forage at various specific locations, such as different foraging heights and microhabitats (Orłowski and Karg 2013; Styring et al. 2016; Mansor and Ramli 2017). The great diversity of prey resources consumed by certain babbler species (e.g., the BTB, MB, and CWB) may also reduce any active dietary competition. Prey species diversity across the 10 babbler species as measured by Shannon Index (H′) values fell within the expected range (typically 1.5–3.5 and <4.5 H′) (Magurran 1988; Table 1). The consumption of greater proportions of diverse prey groups and dietary generalism could also aid in reducing dietary competition at the intrapopulation level (Quevedo et al. 2009; Sherry et al. 2016), especially when foods are abundant. Such diet variability allows sympatric species to adapt successfully to prey availability in particular area. These results support our hypothesis that certain insectivorous birds are generalists with respect to taxonomy to reduce interspecific

competition. We suggest that if the decline in the populations of the generalists is related to food supply, it would most likely be due to a broad-scale decline in insect prey abundance across taxa rather than changes in the availability of specific prey groups.

It is thought that insectivores' diets may respond to arthropod population fluctuations (Clare et al. 2011). Insectivores may be able to be more selective in their diets when insect abundance rises during the wet season (Koselj et al. 2011). Insect abundance and composition are believed to be highly variable in time, and their fluctuations are potentially associated with the amount of rainfall (Borghesio and Laiolo 2004). The collection of dietary samples in our study occurred during rainy seasons (from October to January), thus explaining the low diversity or specialization of prey groups consumed by certain specialist babbler species (e.g., the WCB, BCB, and FB). The wet season is thought to be linked with breeding season may limit foraging range and time of certain birds (Nwaogu et al. 2017). Such specialization in some babblers was consistent with the optimal foraging theory (Emlen 1966), which predicts that predators are selective when faced with abundant prey. This assumption relies on morphological adaptation (e.g., their bill size and shape, wings, and tail) and foraging specialization allowing species to coexist in the same habitat through niche partitioning (Orłowski and Karg 2013; Emrich et al. 2014; Styring et al. 2016; Mansor and Ramli 2017).

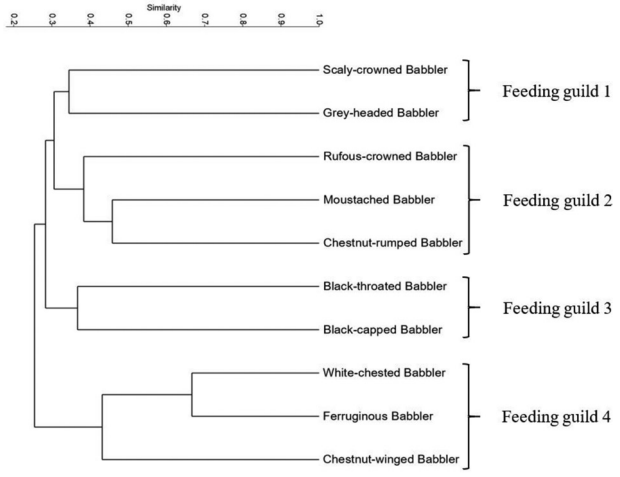

Figure 4. Interspecific dietary relationships of the 10 babbler species based on the occurrence of the prey taxa consumed using Bray–Curtis index cluster analysis. The analysis effectively divided the10 babbler species into 4 main feeding guilds. The cutoff was set at 0.33 similarity. The WCB and FB had the highest dietary similarity index.

However, minimal dietary overlap among sympatric species may also occur, particularly when they sharing similar resources (Mansor et al. 2018). Most of the studied babblers were specialists, and regular or occasional users of aerial leaf litter in Malaysian tropical forests (Mansor et al. 2019), but they favored different strata, ranging from just above the ground up to 8 m above the ground (Mansor and Ramli 2017), causing them to have only slight dietary overlap. The slight dietary overlap in some babblers could also be because of their frequent foraging in mixed-species flocks (Mansor et al. 2020b). Sharing information on foraging places (Waite and Grubb 1988) and feeding on arthropods flushed by other bird species in a flock (Martínez and Robinson 2016) may have led the babblers to feed on similar arthropod groups. For example, the BTB and MB, which have been seen participating in mixed-species flocks, both consumed a relatively similar prey taxa (Table 1). Habit to forage in same height strata could also contribute to dietary overlap, for example, BTB and BCB (Figure 4) used lower vertical strata (Mansor and Ramli 2017) that may lead them to consumed similar prey taxa. Three babbler species grouped in "feeding guild 2" (Figure 4) were also believed to foraged on same vertical strata and used similar foliage density cover (Mansor and Ramli 2017).

By simultaneously using HTS for diet analysis and identifying the arthropods in most of the babblers' preferred microhabitats (i.e., aerial leaf litter; Mansor et al. 2019), we observed evidence for a degree of prey selection. Our study demonstrated that Diptera, Lepidoptera, and Coleoptera were among the dominant food sources for all the studied babbler species (Figure 2), which corresponded with their strong preference for aerial leaf litter. Dipteran and lepidopteran larvae play an important role in decomposition by feeding on decaying organic material (Merritt and Lawson 1992; Hohn and Wagner 2000), and coleopterans hide by aggregating inside aerial leaf litter during the daytime roosts (Greenberg 1987). Certain lepidopteran families (e.g., Geometridae and Noctuidae) pupate in hardened pupal cases hidden among leaf litter (Dugdale 1996). These results confirmed the specialization of the babbler species with respect to aerial leaf litter, whereas the untallied prey taxa found in the birds' fecal

Table 3. Dietary relationships across the 10 babbler species using Spearman correlation rank

	BCB	WCB	FB	SCB	RCB	MB	GHB	BTB	CRB	CWB
BCB	—	0.42848	0.001784	0.23007	0.34035	0.13734	0.15773	0.012214	0.83345	0.99293
WCB	0.089188	—	0.00001	1.00000	0.1486	0.51478	0.82135	0.58715	0.52963	0.000196
FB	0.3419	0.62663	—	0.80497	0.14306	0.2284	0.21582	0.81921	0.14306	0.008105
SCB	0.13484	0	0.027864	—	0.34116	0.1215	0.046869	0.76954	0.34116	0.20945
RCB	0.1073	0.16196	0.16417	0.10712	—	0.096775	0.34035	0.22366	0.22301	0.37
MB	0.16652	0.073422	0.13532	0.17344	0.1858	—	0.029309	0.32345	0.026849	0.83811
GHB	0.15844	0.025482	0.13902	0.22152	0.1073	0.24229	—	0.23713	0.83345	0.47762
BTB	0.27726	0.061225	0.025792	0.033058	0.13669	0.1111	0.13284	—	0.75363	0.87431
CRB	0.02373	0.070855	0.16417	0.10712	0.13689	0.246	0.02373	0.035413	—	0.74595
CWB	0.001	0.40235	0.29227	0.14095	0.10092	0.023057	0.080022	0.017853	0.036554	—

samples were possibly consumed at different foraging locations (i.e., vertical strata; Mansor and Ramli 2017). Certain bird species may forage disproportionately, reflected by their plasticity in foraging heights and substrate utilization when joining heterospecific groups (Farine and Milburn 2013), may lead to the consumption of different varieties of prey taxa. Ultimately, our results suggest DNA metabarcoding can explain where birds are most likely to forage and what they have eaten.

Despite its effectiveness in identifying numerous prey taxa in the diets of insectivores, HTS has some restrictions. Six OTU sequences led to multiple similar species-level identifications and shared DNA barcodes according to the reference database. These situations may have occurred among closely related species or because of the failure of the short-targeted regions used here to discriminate unique prey species (Pompanon et al. 2012; Clare et al. 2014). It has been shown that molecular approaches provide information on which arthropod species are consumed by insectivores, but do not allow the estimation of the actual proportions of prey taxa (Wong et al. 2015). Primer bias can lead to the over- or under-representation of certain taxa in sequencing results (Pompanon et al. 2012). Blattodea were not detected in diets of the studied babblers, although they were found in the morphological dietary analysis (Mansor et al. 2018). However, HTS is highly effective in the detection of very small and soft-bodied arthropods (i.e., dipterans lepidopterans and hemipterans), which were absent in Mansor et al. (2018). Unintentionally, secondary prey taxa, which are in the guts of a predator that is consumed, can be detected (Gerwing et al. 2016). This information can either be considered ecological contamination (e.g., when studying food preference) or not (e.g., when looking at the actual intake) as described by Pompanon et al. (2012). However, the most frequently consumed prey items we detected do not consume arthropods, so this bias is likely to be minimal.

Our results demonstrated that reference databases (i.e., GenBank and BOLD-IDS) have the ability to provide identifications mostly to family, and some to genus and species level of arthropods in the diets of tropical forest birds. Prey taxa identification through molecular analysis is very dependent on the quality of the reference database. A comprehensive reference database is required to match unknown sequences to sequences in the database, and even if there is a match, the level of identification of unknown specimens can be limited by the level of taxonomic identification of the reference sequence. Approximately 24% of the OTUs in this study were identified to a low taxonomic level (i.e., the genus or species level). However, because this study is the first molecular diet analysis on sympatric insectivorous forest birds in Southeast Asia, it is believed that family-level identification is more than sufficient. Currently, there are many initiatives worldwide sequencing arthropods, and in the future, a higher percentage of genera and species-level identification are expected.

Few sequences from the fecal samples matched the reference database of our previous study (24.7%; Mansor et al. 2019). This indicates that some prey items were collected but were not successfully sequenced or that some prey items were not collected in our previous study. The sampling of clusters of curled, dead leaves suspended on vegetation by Mansor et al. (2019) was not comprehensive; these leaves were only examined from 0.5 to 2.0 m, potentially limiting the arthropod taxa found in the sampled litter. The patchy distribution of aerial leaf litter and the heterogeneity of the Krau Wildlife Reserve landscape may also limit the sampling rate. Conducting thorough vertical and horizontal aerial leaf litter sampling could improve measures of prey availability, but such sampling was not possible in this study due to accessibility and time constraints. Although the sample size is relatively low, the detection of broad prey taxa in our study suggests that HTS approach could act as an important tool for describing food web structures and complex species interactions. Larger sample size may provide a more complete dietary assessment that organizes the structure for the bird community in the wild. Despite the advantages and disadvantages of these sophisticated techniques, this study is a great example of how rapid molecular dietary screening can produce valuable and broad-ranging ecological data for the formulation of biodiversity and conservation programs.

Acknowledgments

We thank the Department of Wildlife and National Parks, Hassan Kassim, Mohamad Nazli Mahamad Zain, Aziz Che Man, and Khairul Nizam Kamaruddin for their help and permission to perform this research in the Krau Wildlife Reserve. We also thank Muhd Fadhil Abdul Rasid, Muhammad Rasul Abdullah Halim, and Mohd Sanusi Mohamed for field assistance. None of the authors has a conflict of interest associated with this manuscript. The collection of fecal samples was permitted by the Department of Wildlife and National Parks Peninsular Malaysia under special permit [JPHL&TN(IP):60-4/1.13 JILID 18 (108)].

Funding

This study was funded by the Fundamental Research Grant Scheme (FRGS), Ministry of Higher Education Malaysia, under grant FRGS/1/2020/STG03/UKM/02/5.

Conflicts of Interest Statement

The authors have no conflicts of interest to declare.

Supplementary Material

"Supplementary material can be found at https://academic.oup.com/cz".

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