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Research Article

Comparison of butterflies, bats and beetles as bioindicators based on four key criteria and DNA barcodes

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Abstract
Informed conservation decision-making requires meaningful biodiversity assessment, yet performing an inventory of all species present at a site is an impossibility. A small group of species is frequently used as a proxy for "total" biodiversity, and various attributes required by bioindicator groups have been suggested. We synthesized these suggestions as four key criteria: i) tractable taxonomy, ii) easily surveyed, iii) broadly distributed higher taxa but specialized species, and iv) diversity patterns reflected in other groups; and compare three groups - bats, beetles and butterflies - against these criteria to evaluate their potential as bioindicators in Malaysia. DNA barcodes from butterflies, bats and beetles sampled during standardized surveys at Rimba Ilmu Botanic Garden and Ulu Gombak Forest Reserve were sorted into molecular operational taxonomic units (MOTU) and assigned species and family names. Beetle and butterfly sampling required a similar number of person-hours per species, which was an order of magnitude lower than that required for bat sampling. It was easier to generate DNA barcodes for butterflies and bats than for beetles and the number of MOTU assigned a species and/or family name was higher for butterflies and bats (>82%) than for beetles. Most butterfly and bat families were found at both sites but the species of all three groups showed little overlap between sites (<15%). The species richnesses of all three groups were correlated with each other, but only bat and butterfly species richness was strongly correlated and statistically significant. Based on the four criteria, butterflies showed greatest potential as bioindicators.

Keywords: Chiroptera, Coleoptera, DNA barcoding, Lepidoptera, bioindicators

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Introduction

The world is facing rapid growth of the human population and widespread urbanization [1,2]. In Asia in particular, the human population has doubled over the last 40 years [3]. Consequently, the availability of habitats for wildlife is diminishing, resulting in extirpation of species [4,5]. Protecting habitats is vital to conserve populations of species in decline, yet the designation of all remaining wild land as protected sites is unrealistic. In order to conserve the most species, sites with the highest total biodiversity should be selected to receive the toughest protection [6]. Informed decision-making requires assessment of the biodiversity of a site (α-diversity) and comparisons of biodiversity between sites (ß-diversity) [7]. Because performing an inventory of all the species present at a site is impossible due to limited time and resources, a relatively small group of species, sometimes even a single species [8], is frequently used as a proxy for "total" biodiversity [9-11].

Various criteria have been suggested for the selection of biodiversity "indicator" groups (e.g. [9,12-14]) but the attributes commonly regarded as essential can be synthesized under four key criteria:

(i) *Tractable taxonomy* – The species must be easy to identify, even by non-specialists, facilitating comparisons between surveys conducted at different times, in different locations and by different researchers. DNA barcoding, the use of short standardized DNA sequences for species recognition, can impact on this criterion by allowing rapid recognition of interoperable molecular taxonomic units (species) by non-experts [15] (but see [16]).

(ii) *Easily surveyed* – A well-known ecology allows for the design of effective sampling protocols that can be standardized and deployed in a cost- and time-effective manner.

(iii) *Broadly distributed higher taxa; specialized and habitat-sensitive lower taxa* – The group must be present at all sites with stable population sizes, but exhibit different species composition at different sites.

(iv) *Patterns of biodiversity reflected in other groups* – The group should be a biodiversity "umbrella", meaning conservation of the group would benefit numerous co-occurring species from other groups [13].

Various animal groups have been advocated as useful bioindicators, including: butterflies (Lepidoptera), due to their intimate relationship with plants (e.g. [8,17]); bats (Chiroptera), due to their high diversity, top-predator and conservation status (e.g. [18,19]); and dung beetles (Coleoptera), due to their ecological specialization and relationship with mammals (e.g. [20,21]). In this study we assessed butterflies, bats and beetles against the four key criteria above to evaluate their potential as bioindicator groups in Malaysia.

Methods

Field sites

Standardized surveys of the three target groups (bats, dung beetles and butterflies) were conducted at Rimba Ilmu, University of Malaya, Kuala Lumpur, Malaysia (N 03° 7', E 101° 39') and Ulu Gombak Forest Reserve, Selangor, Malaysia (N 03° 19', E 101° 44') (Fig. 1). Rimba Ilmu is an 80 ha tropical botanical garden, formerly a rubber plantation, which houses over 1,600 species of tropical plants [22]. Ulu Gombak is a 17,000 ha selectively logged reserve forest [23]. The surveys were conducted at each site over three days and three nights (six sampling events) and were completed during two consecutive weeks in March 2013. The days were all dry and sunny and the nights also clear and dry, with the exception of a small amount of rain on the second night at Rimba Ilmu (<2 h).

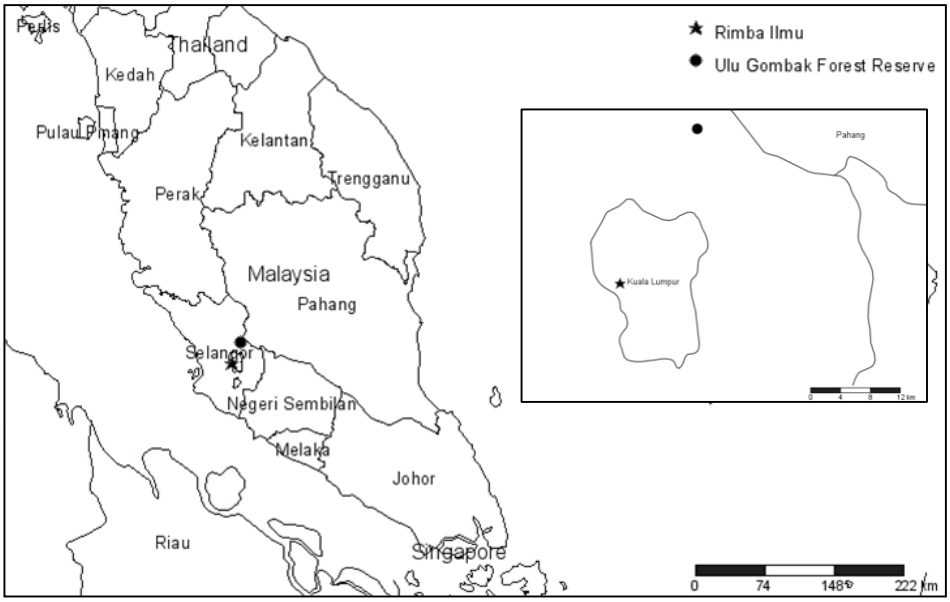

Fig. 1. The two study sites in Peninsular Malaysia.

Standardized sampling protocols

Our study was approved by the Department of Wildlife and National Parks (DWNP) Peninsular Malaysia and the University of Malaya Institutional Animal Care and Use Committee (UMIACUC).

Butterflies were sampled using sweep nets by two experienced butterfly catchers walking continuously at a standardized pace along two 1,000 m transects, 500 m apart, between 10:00 and 12:00 (following [24]). The right hind leg (when viewed dorsally) of each captured butterfly was collected into a 1.5 ml tube using forceps before the butterfly was released. If a butterfly with no right hind leg was captured it was released as a probable re-capture.

Ten mist nets and four harp traps were set along two transects 500 m apart between 19:00 and 07:30 to sample bats. Traps were checked every hour and a small wing punch was collected from each captured bat into a 1.5 ml tube following AMNH [25]. If a bat with a wing punch was captured it was released without re-sampling as a probable re-capture.

Beetles were sampled overnight using the standardized trapping protocol of Inward et al. [26] with slight modifications. In brief, 20 baited pitfall traps were set 10 m apart along two 90 m transects, 500 m apart. On each transect, five traps were baited with fresh cow dung and five with raw chicken liver. Traps were emptied each morning. Beetles were rinsed in ddH_2O then complete specimens of small beetles, and single legs of large beetles, were placed individually into 1.5 ml tubes.

DNA barcoding

DNA extraction from bats and beetles was performed using a Nucleospin kit (Machery-Nagel, Germany) and from butterflies using a XytXtract Animal kit (Xytogen, Australia) following the manufacturers' instructions. A first attempt was made to PCR amplify the DNA barcode region of COI mtDNA following standard protocols [27] using the primer pairs LepF1/LepR1 for butterflies and beetles and VF1d_t1/VR1d_t1 for bats [28]. If the first PCR failed PCR

troubleshooting was conducted using the primer pairs MLepF1/LepR1 [27] for butterflies and beetles and RonM/VF1d_t1 [28] for bats. PCR products were sequenced using LepR1 or the M13R (t1) tail. The DNA barcodes were edited and aligned [27] and sorted into molecular operational taxonomic units (MOTU) using the online Automatic Barcode Gap Discovery (ABGD) system [29]. Previous studies have shown that there is typically a distinct pattern to intra- and interspecies DNA barcode genetic distances, a "barcode gap", but that this pattern can be unique to a dataset. ABGD uses an automatic recursive procedure to converge on the best patterns for the dataset and arranges DNA barcodes into clusters accordingly. The median number of ABDG clusters was used as the basis for our MOTU as this has produced good correspondence with traditional species in empirical studies [30].

Representatives of each MOTU were submitted to the full database of the BOLD identification engine [31] to assign a taxonomic name to the MOTU. Species names were assigned using a >98% sequence similarity threshold. When there was no match >98%, family names were assigned using the strict tree-based method of Wilson et al. [32] based on the "Tree Based Identification" of the BOLD identification engine [31]. This method requires the unknown DNA barcode to be nested within a cluster of sequences all from the same family.

Assessment of the groups against key criteria

(i) *Tractable taxonomy* – This criterion was assessed based on DNA barcoding success. Successful PCR amplification on the first pass and the number of MOTU assigned taxonomic names were quantified.

(ii) *Easily surveyed* – The number of individuals and MOTU sampled were divided by the total number of person-hours required for surveying the group.

(iii) *Broadly distributed higher taxa; specialized and habitat-sensitive lower taxa* – The similarity between sites in terms of higher taxa (families) and species (MOTU) was assessed using the Sorenson Similarity index. The index has values between 0 and 1, with 1 indicating the sites are identical. For families, values closer to 1 are preferable, whereas for species, values closer to 0 are preferable.

(iv) *Patterns of biodiversity reflected in other groups* – The relationship between the species richnesses of each group was analyzed using Pairwise Spearman's Rank Correlation (following [11]).

Results

Tractable taxonomy

The PCR success rate on the first pass was high for both bats and butterflies (>70%) but low for beetles (36%) (Table 1). After troubleshooting, eighteen butterfly DNA barcodes were discarded as likely contaminants as they were either messy sequences or failed to match target taxa in BOLD. Two bat samples failed to PCR amplify after several attempts. Two beetle samples also failed to PCR amplify after several attempts while a further three were likely contaminants as they showed high similarity with non-target taxa. The DNA barcodes produced for this study are available on BOLD [31] in the public dataset DS-MYBIO (http://www.boldsystems.org/index.php/Public_SearchTerms?query=DS-MYBIO). The number of MOTU assigned a species and/or family name was higher for butterflies and bats (>82%) than for beetles (Table 1).

Table 1. DNA barcoding success for butterflies, bats and beetles.

Group	n[a] (Rimba Ilmu/Ulu Gombak)	PCR success first pass (%)	Successfully barcoded after troubleshooting (%)	Number of MOTU	Number of families	MOTU assigned a species name (%)	MOTU assigned a family name (%)
Butterflies	125/138	71	93	78	6[b]	82	99
Bats	16/27	81	95	7	3	86	100
Beetles	123/93	36	98	40	10	8	68

[a]Includes samples which failed to amplify and likely contaminants.
[b]There are only six families of butterflies, but one specimen of the Lepidoptera family Callidulidae, which contains day-flying moths, was also sampled as part of this indicator group.

Easily surveyed

Our study required 216 person-hours for sampling bats, 24 for sampling butterflies and 14 for sampling beetles. Bats accounted for an order of magnitude fewer individuals and species sampled per person-hour than the beetles and butterflies (Fig. 2).

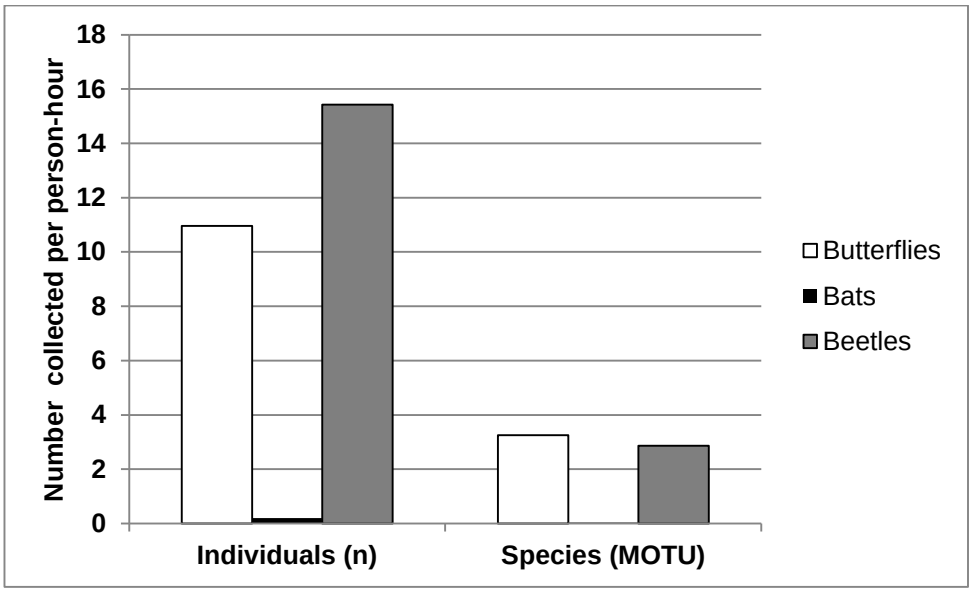

Fig. 2. Comparison of effort required to sample the three potential bioindicator groups.

Broadly distributed higher taxa; specialized and habitat-sensitive lower taxa

Rimba Ilmu and Ulu Gombak Forest Reserve showed high similarity (≥80% shared between the two sites) in terms of the bat and butterfly families sampled. All groups appeared relatively habitat-sensitive at species level with less than 15% overlap of MOTU between the two sites (Fig. 3).

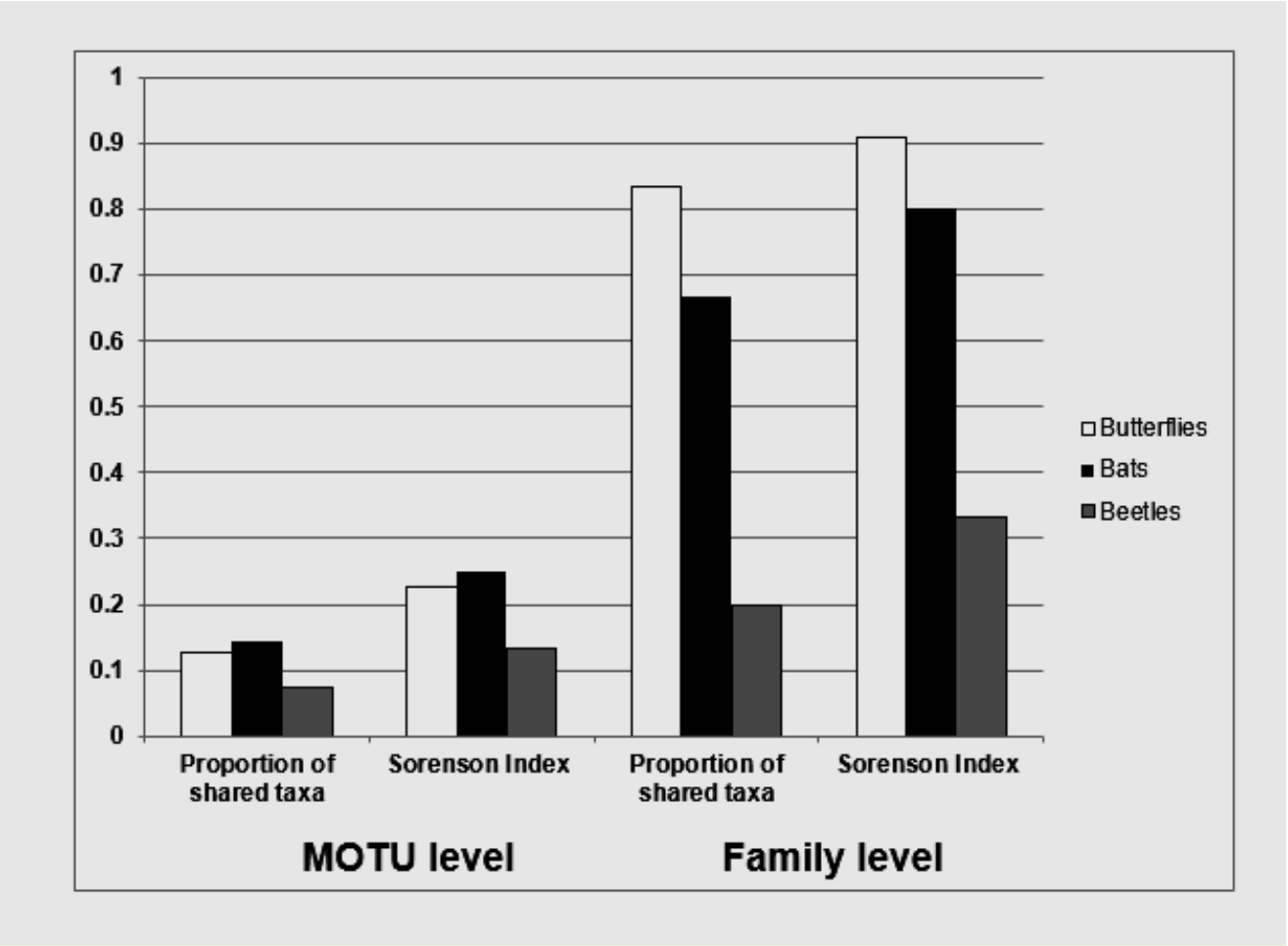

Fig. 3. The distribution of butterfly, bat and beetle taxa between two sites, Rimba Ilmu and Ulu Gombak

Patterns of biodiversity reflected in other groups

The species richnesses of all three groups were positively correlated with each other (Table 2; Fig. 4). The species richnesses of bats and butterflies were strongly correlated and statistically significant ($p < 0.02$). Both bat and beetle species richnesses and beetle and butterfly species richnesses were weakly correlated and not statistically significant (Table 2).

Table 2 Pairwise comparisons using Spearman's Rank Correlation between species diversity of butterflies, bats and beetles during six sampling events at Rimba Ilmu and Ulu Gombak. Values below the diagonal are the Spearman's Rank Correlation coefficient; values above the diagonal are p-values.

	Butterflies	Bats	Beetles
Butterflies		<0.02	<0.32
Bats	0.88		<0.82
Beetles	0.49	0.12	

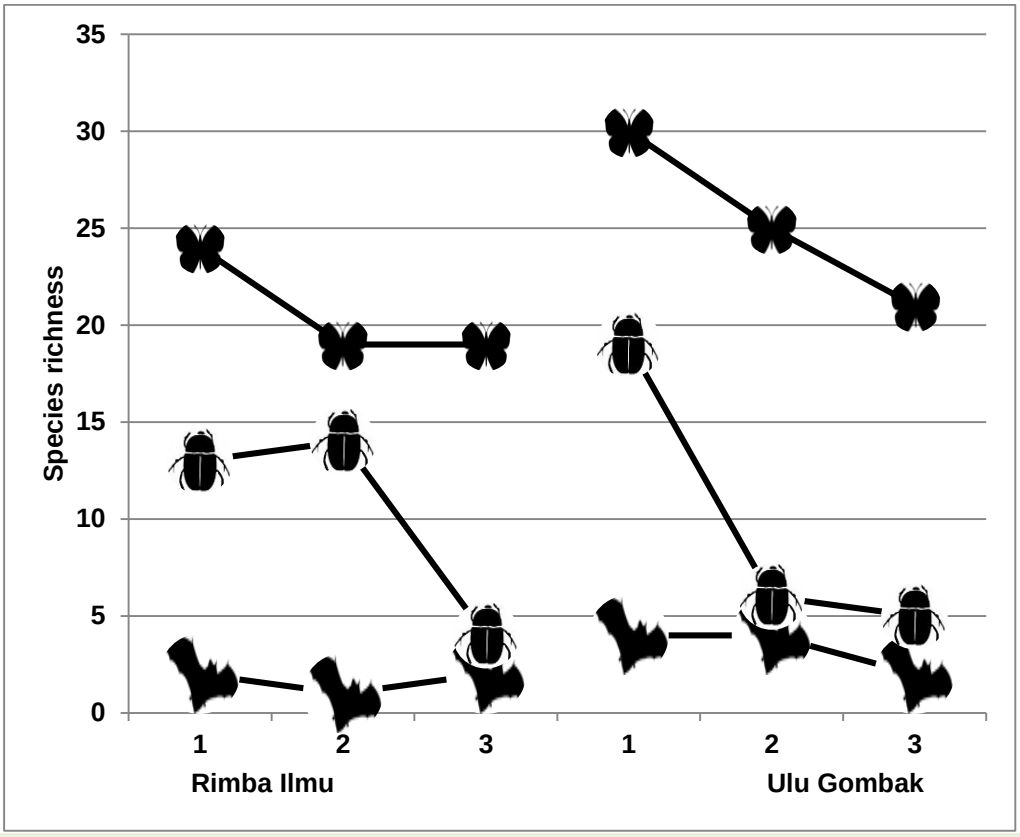

Fig. 4 Patterns of species richness of butterflies, bats and beetles during six sampling events at Rimba Ilmu and Ulu Gombak. The relationship between the species richnesses of each group was analyzed using pairwise Spearman's rank correlation.

Discussion

The first criterion used to assess bioindicator potential was "taxonomic tractability"; the ease of identifying species by non-experts. This criterion was evaluated through DNA barcoding success. Butterflies and bats have been the target of large DNA barcoding campaigns (e.g. [33-35]) including recently in Southeast Asia [36,37]. Consequently the protocols for DNA barcoding these groups are well-optimized [27,38]. Therefore it was not surprising that bats had high PCR amplification success in our study (81% on the first pass) but the lower success (71%) for butterflies was unexpected. The DNA barcode reference libraries for Lepidoptera and Chiroptera are well-developed with more than 700,000 DNA barcodes (75,000 species) for Lepidoptera and 20,000 DNA barcodes (700 species) for Chiroptera in BOLD [31]. The large reference libraries resulted in a high number (>82%) of assignments of species and family names to the MOTU from these groups, after "blasting" representatives against BOLD. An advantage of the DNA barcoding approach is that it can assign samples to "dark taxa," adding precision to species inventories. "Dark taxa" are species that have been previously recognized and reported by researchers, often through DNA barcoding, but which have not (yet) been formally described [39]. For example, *Cynopterus* cf. brachyotis Forest, also known as *Cynopterus* JLE sp.A, reported by Francis et al. [36] was recorded in this study. Unlike bats and butterflies, beetles have relatively poor coverage in BOLD and the lack of optimization for beetle DNA barcoding is a major drawback to the use of beetles as a bioindicator group. DNA barcoding studies of beetles have tended to target the 3' end of COI mtDNA [40] and the commonly used 'Lep' insect primers targeting the 5' "barcode region" [27], as used by us, seem to have low success for beetles. However, new primers have recently been designed to target the 5' region in beetles [41] which could impact the rating of beetles for this criterion (Table 3).

Table 3 Ranking of groups for bioindicator potential according to four key criteria.

Criterion	Butterflies	Bats	Beetles
Tractable taxonomy	2	1	3
Easily surveyed	2	3	1
Taxonomic distribution	1	2	3
Diversity patterns reflected in other groups	1	2	3
Overall Rank	**1**	**2**	**3**

The second criterion used to assess bioindicator potential of a group was "easily surveyed" and was quantified through the cost- and time-effectiveness of the sampling protocol. In terms of person-hours required for our sampling protocols, beetles required the fewest hours while bats required the most. Bat sampling requires at least two people to set up and disassemble mist nets and harp traps, as well as to attend to the catch regularly to avoid escapees and injury to the bats. Conversely, beetle traps can be set by an individual and left unattended overnight. In terms of cost, bat sampling requires expensive specialized equipment, while butterflies and beetles can be sampled with inexpensive homemade devices. However, it is also worth considering the ease of achieving a precisely comparable sampling protocol. For example, we can easily imagine a tendency for keen butterfly collectors to target any "rare" or unusual

species they encounter during their walks rather than collecting randomly from among the butterfly assemblages. An alternative approach to sweep net sampling could be to use Malaise or light traps to sample lepidopterans, which may also reduce the required person-hours. The pitfall traps were raided by dogs at Ulu Gombak Forest Reserve but left undisturbed at Rimba Ilmu. Ants were also a confounding factor, as they built nests over the traps at Ulu Gombak Forest Reserve and probably contributed to the lower number of beetles sampled at this site. The choice of location for setting mist nets and harp traps can affect the efficiency of bat trapping, being influenced by vegetation and microclimate [42]. Other factors affecting the ability to generate comparable survey data include 'tourists' and the seasonality of species [43].

The third criterion used to assess bioindicator potential of a group was "broadly distributed higher taxa; specialized and habitat-sensitive lower taxa". There was a high similarity (>66%) of family composition at Rimba Ilmu and Ulu Gombak Forest Reserve for both bats and butterflies. Bats are generally widespread in term of distribution but each species occupies a specific habitat (e.g. caves, bamboos, hollow barks, foliages etc.) [44]. Butterflies are ubiquitous in vegetated terrestrial ecosystems yet specialized, e.g. to disturbed or primary forest areas, as their caterpillars depend exclusively on specific host plants [45]. Our sampling included ten families of beetles and the group exhibited a pattern of specialization at both low and high taxonomic levels, although Scarabaeidae dominated the samples at both sites, indicating their high preference for the baits [46].

The fourth criterion used to assess bioindicator potential of a group was "patterns of biodiversity reflected in other groups". A bioindicator group should be able to be used to predict the diversity patterns of other unrelated groups at the site. We found that the species richnesses of all three groups were correlated with each other. The species richness of butterflies had a significant correlation with the species richness of bats suggesting that the species richness of butterflies is useful to predict the species richness of bats and vice-versa. Harvey et al. [47] likewise found a significant correlation between species richness of bats and nectarivorous butterflies in Rivas, Nicaragua. However, in another study in the Neotropics, butterfly species richness was strongly correlated with species richness of birds but not with mammals [48]. Under this criterion we rank butterflies higher than bats as the butterflies-beetles correlation was marginally stronger than the bats-beetles correlation (Table 2).

Implications for conservation

In light of rapid habitat loss in Southeast Asia, there is a pressing need for a standardized system of rapid, yet meaningful, measures of biodiversity. Considering that performing a complete inventory of species at a site is an impossibility, it is not surprising that research on the use of small groups of species as indicators of biodiversity has a long history (e.g. [12,49-51]). However, the choice of such indicator groups still remains largely intuitive rather than evidence-based [52]. We present a model for assessment of the bioindicator potential of a group based on four key criteria, which we then used to provide quantitative data on the bioindicator potential of three groups (bats, beetles and butterflies) surveyed at two sites in Malaysia.

We found that butterflies had the most potential as a bioindicator group, ranking first in two of the criteria - taxonomic distribution and reflection of diversity patterns in other groups, and second in the other two - taxonomic tractability and ease of surveying (Table 3). DNA barcoding protocols for butterflies are well-optimized and there is a well-developed DNA barcode reference library available with which to assign butterfly DNA barcodes a precise taxonomic name. Furthermore, butterfly sampling requires only a few hours per day with simple apparatus. Butterfly families are few and widespread, but species are habitat specific. Butterfly species richness showed a significant correlation with the species richness of bats. The ability to generate comparable survey data is an important factor in the choice of bioindicator groups, as is the development of optimized DNA barcoding protocols and DNA barcode reference libraries.

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