NRC Research Press

ARTICLE

Field calibration of blowfly-derived DNA against traditional methods for assessing mammal diversity in tropical forests[1]

Ping-Shin Lee, Han Ming Gan, Gopalasamy Reuben Clements, and John-James Wilson

Abstract: Mammal diversity assessments based on DNA derived from invertebrates have been suggested as alternatives to assessments based on traditional methods; however, no study has field-tested both approaches simultaneously. In Peninsular Malaysia, we calibrated the performance of mammal DNA derived from blowflies (Diptera: Calliphoridae) against traditional methods used to detect species. We first compared five methods (cage trapping, mist netting, hair trapping, scat collection, and blowfly-derived DNA) in a forest reserve with no recent reports of megafauna. Blowfly-derived DNA and mist netting detected the joint highest number of species (n = 6). Only one species was detected by multiple methods. Compared to the other methods, blowfly-derived DNA detected both volant and non-volant species. In another forest reserve, rich in megafauna, we calibrated blowfly-derived DNA against camera traps. Blowfly-derived DNA detected more species (n = 11) than camera traps (n = 9), with only one species detected by both methods. The rarefaction curve indicated that blowfly-derived DNA would continue to detect more species with greater sampling effort. With further calibration, blowfly-derived DNA may join the list of traditional field methods. Areas for further investigation include blowfly feeding and dispersal biology, primer biases, and the assembly of a comprehensive and taxonomically-consistent DNA barcode reference library.

Key words: camera traps, cage traps, hair traps, Malaysia, mist nets.

Résumé : La mesure de la diversité des espèces de mammifères fondées sur l'analyse de l'ADN extrait d'invertébrés a été avancée comme alternative aux méthodes traditionnelles. Cependant, aucune étude de terrain n'a été réalisée pour comparer les deux simultanément. Dans la péninsule malaisienne, les auteurs ont comparé la performance de l'ADN de mammifères extrait de mouches vertes et bleues (Diptera : Calliphoridae) à celle des méthodes traditionnelles pour détecter les espèces. Cinq méthodes (trappes, filets japonais, pièges à poils, collecte de fèces et l'ADN dérivé de mouches) ont été comparées au sein d'une réserve forestière où aucune mégafaune n'avait été rapportée récemment. L'ADN dérivé de mouches et les filets japonais ont permis de détecter le plus grand nombre d'espèces (n = 6). Seule une espèce a été détectée au moyen de plusieurs méthodes. Comparée à d'autres méthodes, seule l'analyse d'ADN dérivé de mouches a permis de détecter à la fois des espèces volantes et non-volantes. Dans une autre réserve forestière, riche en mégafaune, l'analyse d'ADN extrait de mouches a été comparée aux pièges photographiques. L'analyse d'ADN a permis de détecter plus d'espèces (n = 11) que les pièges photographiques (n = 9), une seule espèce ayant été détectée par les deux méthodes. La courbe de raréfaction suggère que l'analyse d'ADN dérivé de mouches pourrait continuer à détecter plus d'espèces en augmentant l'effort d'échantillonnage. Suite à davantage de travaux de calibration, l'analyse d'ADN extrait de mouches pourrait s'ajouter à la liste des méthodes de terrain traditionnelles. Des investigations additionnelles pourraient se pencher sur l'alimentation et la dispersion des mouches, les biais dus aux amorces et la production d'une banque de codes à barres de l'ADN de référence qui soit à la fois exhaustive et juste sur le plan taxonomique. [Traduit par la Rédaction]

Mots-clés : pièges photographiques, trappes, pièges à poils, Malaisie, filets japonais.

Received 30 November 2015. Accepted 17 April 2016.

Corresponding Editor: Sarah Adamowicz.

P.-S. Lee and J.-J. Wilson.* Museum of Zoology, Institute of Biological Sciences, Faculty of Science, University of Malaya, 50603 Kuala Lumpur, Malaysia; Ecology and Biodiversity Program, Institute of Biological Sciences, Faculty of Science, University of Malaya, 50603 Kuala Lumpur, Malaysia.
H.M. Gan. School of Science, Monash University Malaysia, Jalan Lagoon Selatan, Bandar Sunway, 47500 Petaling Jaya, Selangor, Malaysia; Monash University Malaysia Genomics Facility, Monash University Malaysia, Jalan Lagoon Selatan, Bandar Sunway, 47500 Petaling Jaya, Selangor, Malaysia.
G.R. Clements. School of Science, Monash University Malaysia, Jalan Lagoon Selatan, Bandar Sunway, 47500 Petaling Jaya, Selangor, Malaysia; Centre for Tropical Environmental and Sustainability Science, and College of Marine and Environmental Sciences, James Cook University, 4870 Cairns, Queensland, Australia; Kenyir Research Institute, Universiti Malaysia Terengganu, 21030 Kuala Terengganu, Malaysia; Rimba, 4 Jalan 1/9D, 43650 Bandar Baru Bangi, Selangor, Malaysia.
Corresponding author: John-James Wilson (email: wilso04@gmail.com).
*Present address: International College Beijing, China Agricultural University, Beijing 100083, P.R. China.
[1]This paper is part of a special issue entitled Barcodes to Biomes.

Introduction

A wide variety of field methods have been used to assess mammal diversity in tropical forests. Frequently used methods include live trapping, such as cage traps (Hanif-Ridzuan et al. 2010; Madinah et al. 2011), mist nets, and harp traps (Kingston et al. 2003; Sing et al. 2013), camera traps (Clements 2013; Hedges et al. 2015*a*), indirect signs such as tracks or scat (Daim 2002), interviews with local communities (Sharma et al. 2005), direct observations by researchers (Syakirah et al. 2000; Jayaraj et al. 2013), and hair traps (Castro-Arellano et al. 2008; Hedges et al. 2015*b*). Recent additions to the toolbox are secondary sources of mammal DNA, for example, mammal DNA detected from owl-pellet bones (Rocha et al. 2015) and invertebrate gut contents (Calvignac-Spencer et al. 2013*a*, 2013*b*; Schnell et al. 2012, 2015; Lee et al. 2015). These methods can provide accurate identification of mammal species, are not stressful to the mammals themselves, require the least ecological and taxonomic expertise, and yet have the potential to detect rare and cryptic species (Calvignac-Spencer et al. 2013*a*; Schnell et al. 2012; Lee et al. 2015).

Blowflies (Diptera: Calliphoridae) have shown promising potential as sources of mammal DNA due to their presence in all habitats (Norris 1965) and broad host preferences as saprophagous and coprophagous generalists (Calvignac-Spencer et al. 2013*a*, 2013*b*; Azwandi et al. 2013; Lee et al. 2015; Schnell et al. 2015). *Chrysomya bezziana* has been reported feeding on mammal species from the orders Artiodactyla (7 spp.), Carnivora (6 spp.), Diprotodontia (2 spp.), Perissodactyla (4 spp.), Proboscidea (1 spp.), and Primates (1 spp.) at a zoo in Malaysia (Spradbery and Vanniasingham 1980). *Lucilia sericata* is found in Asia, North America, and Europe (James 1947; Picard and Wells 2010), and it has been reported to feed on wounds of sheep, cats, a dog, and a horse in Israel (Schnur et al. 2009). Blowflies, such as *Chrysomya megacephala*, were the first and most abundant insects arriving at *Macaca fascicularis*, *Oryctolagus cuniculus*, and *Rattus norvegicus* carcasses in a forest in Peninsular Malaysia (Azwandi et al. 2013). Standardised methods for sampling of blowflies have been developed that take into account the persistence period of mammal DNA in blowfly guts (Lee et al. 2015). Despite the increasing interest in invertebrate-derived mammal DNA for mammal diversity assessments, field calibrations of the performance of invertebrate-derived mammal DNA against traditional methods have yet to be conducted (Schnell et al. 2015).

Each field method listed above differs in terms of the targeted mammal groups and resource costs, with advantages and disadvantages for each method (summarised in Table 1). Recently, a number of studies from the forests of Peninsular Malaysia, home to 222 mammal species (DWNP 2010), have simultaneously tested the effectiveness of different methods in detecting mammal species (Syakirah et al. 2000; Jayaraj et al. 2012, 2013; Tingga et al. 2012). In Gunung Stong State Park, Kelantan, a combination of cage traps, mist nets, and harp traps yielded low capture rates of non-volant small mammals (Jayaraj et al. 2012); cage traps did not detect treeshrews and squirrels (probably due to bait incompatibility and poor positioning), while harp traps captured a low diversity of insectivorous bats. In Taman Negara, Pahang, a combination of cage traps, Sherman traps, mist nets, and harp traps demonstrated the efficiency of harp traps for mammal sampling (Tingga et al. 2012). The harp traps accounted for 84% of the bat species reported and 65% of the total mammal species. In contrast, at Wang Kelian State Park, Perlis, after several years of surveys using a combination of harp traps, cage traps, and direct observations, the inclusion of mist nets in the latest sampling period increased the number of species sampled by 33% (from an average of 24 per survey to 36; Jayaraj et al. 2013).

Here, we evaluate the potential of blowfly-derived DNA for mammal diversity assessments in tropical forests. We conducted two field studies to calibrate the performance of blowfly-derived mammal DNA at generating species inventories and richness estimates against traditional methods in Peninsular Malaysia. We first compared five methods—cage trapping, mist netting, hair trapping, scat collection, and blowfly-derived DNA—in a tropical forest reserve with no recent reports of megafauna. Next, we compared blowfly-derived DNA with the most popular method to assess megafauna diversity—camera traps—in another tropical forest reserve with megafauna.

Materials and methods

Study sites

Two field calibrations were conducted in two forest reserves in Peninsular Malaysia—Ulu Gombak Forest Reserve (UGFR) in Selangor and Tembat Forest Reserve (TFR) in Terengganu. UGFR comprises 17 000 ha of logged dipterocarp forest (3°20′N, 101°45′E; Fig. 1). UGFR is considered one of the most species-rich localities for Old World bats (Sing et al. 2013) and has been intensively studied since the establishment of Ulu Gombak Field Studies Centre (University of Malaya) 50 years ago (Medway 1966). We compiled an extensive mammal checklist for UGFR based on previous records (including University of Malaya student theses) of sampled or observed species (supplementary data, Table S1[2]). TFR, comprising logged dipterocarp forest (5°11′N, 102°41′E), is one of 17 ecological linkages recognised in the Malaysia Federal Government's "Central Forest Spine Master Plan for Ecological Linkages" to restore connectivity between four fragmented forest complexes (DTCP and DOF 2012).

[2]Supplementary data are available with the article through the journal Web site at http://nrcresearchpress.com/doi/suppl/10.1139/gen-2015-0193.

Table 1. Comparison of different field methods including targeted mammal groups, advantages, disadvantages, and cost.

Field method	Targeted mammal groups	Advantages	Disadvantages	Cost
Cage traps	Rodents, insectivores (Catling et al. 1997)	Operates 24 h without supervision (Catling et al. 1997)	Difficult to standardise (varying dimensions, baited/un-baited, and deployment location effects) (Fontúrbel 2010; Torre et al. 2010)	Medium (Garden et al. 2007)
Mist nets	Frugivorous bats (Stoner and Timm 2004)	Readily portable (Kuenzi and Morrison 1998)	Must be monitored constantly, as bats can become easily entangled and must be freed individually (Kuenzi and Morrison 1998)	Low (Kuenzi and Morrison 1998)
Harp traps	Insectivorous bats (Kingston et al. 2003)	Does not require constant monitoring (Tidemann and Woodside 1978)	Bulky and not easy to transport (Tidemann and Woodside 1978)	High (Tidemann and Woodside 1978)
Camera traps	Medium to large-bodied mammals (Bernard et al. 2013)	Effective in detecting species rarely recorded from live traps or direct observations (e.g., Hose's civet, *Diplogale hosei*; Bernard et al. 2013)	May under-represent species with specific habitats and unable to distinguish closely related species (e.g., muntjac and mouse-deers; Bernard et al. 2013)	High (Sanderson and Trolle 2005)
Indirect signs	Medium to large-bodied ground dwelling mammals (Catling et al. 1997)	Effective in detecting species inhabiting open areas (e.g., otters and ungulates; Catling et al. 1997)	Imprecise in species identification (Davison et al. 2006; Mumma et al. 2014); Accuracy and precision are dependent on field conditions and expertise of identifiers (Silveira et al. 2003)	Low (Garden et al. 2007)
Interviews	Medium to large-bodied mammals (Mohd-Azlan et al. 2013)	No equipment required and can provide important collateral data (Mohd-Azlan et al. 2013)	Less reliable due to varying survey design and bias produced by respondents such as different recalling ability of respondents for different periods of time (Meijaard et al. 2011) (e.g., most villagers interviewed could not recall how many tigers have been killed due to livestock depredation by tigers; Sharma et al. 2005)	Low (Mohd-Azlan et al. 2013)
Direct observations	Medium to large-bodied mammals that are readily observable (Gese 2001)	Can confirm species presence directly and effectively detect rare and endangered species (e.g., black-footed ferrets, *Mustela nigripes*; Gese 2001)	The detection success relies much on the protocols used and expertise of identifiers (Hoppe-Dominik et al. 2011; Roberts 2011)	Low (Garden et al. 2007)
Hair traps	All ground-dwelling mammals (Stanton and Anderson 1998; Castro-Arellano et al. 2008)	Portable (Castro-Arellano et al. 2008); Reliable in identifying species through genetic analysis (Beja Pereira et al. 2009; Mullins et al. 2010)	Different trap types used could influence the detection success (Castro-Arellano et al. 2008) such as the use of hair traps with mechanical devices like barb wire or attached glue-like substances that rarely collect high proportions of hairs with roots (Valderrama et al. 1999; Ebert et al. 2010)	Low (Castro-Arellano et al. 2008)

Table 1 (*concluded*).

Field method	Targeted mammal groups	Advantages	Disadvantages	Cost
Invertebrate-derived mammal DNA	All mammals (Calvignac-Spencer et al. 2013*a*, 2013*b*; Schnell et al. 2012, 2015; Lee et al. 2015)	Can accurately identify rare and cryptic species with least ecological and taxonomic expertise (Calvignac-Spencer et al. 2013*a*; Schnell et al. 2012) (e.g., annamite striped rabbit was recorded from DNA recovered from leeches in Vietnam; Schnell et al. 2012); Blowflies in particular have good potential as sources of mammal DNA due to their presence in all habitats and broad host preferences (Norris 1965)	Blowflies must be collected every 24 h as mammal DNA persists at least 24 h in blowfly guts (Lee et al. 2015)	High (Calvignac-Spencer et al. 2013*a*, 2013*b*; Schnell et al. 2015)
Owl pellet bones-derived DNA	Small mammals (Bonvicino and Bezerra 2003; Torre et al. 2004; Souza et al. 2010; Teta et al. 2010; Rocha et al. 2015)	Effective in detecting and identifying small mammals including those with elusive behaviours and low densities (Rocha et al. 2015) (e.g., two undescribed species of *Oecomys* were recorded from DNA recovered from owl pellet bones of barn owls; Rocha et al. 2015)	The age of pellet remains has an impact on the level of PCR inhibition (Buś et al. 2014); Easily degraded as non-invasive samples in tropical regions due to climatic conditions (e.g., Mukherjee et al. 2010; Vynne et al. 2012)	Low (Rocha et al. 2015)

Field methods 1

Our first field survey was conducted at UGFR between 3 November 2014 and 25 December 2014 for two nights each week (16 nights in total). Two transects of 100 m were established 1500 m apart (Fig. 1). The survey incorporated baited cage traps, mist nets, baited hair traps, scat collection, and baited blowfly traps.

Baited cage traps

Ten wire mesh cage traps (Fig. 1) were set per transect at 10 m intervals, monitored and baited with fresh banana daily. Banana is reported as being among the effective bait for cage traps used for small mammal diversity assessments in tropical forests of Malaysia (Bernard 2003; Payne and Francis 2005; Madinah et al. 2011) and is resistant to removal by invertebrates (Bernard 2003). Cage traps were checked daily, and hair samples from any trapped mammals were collected into a 1.5 mL microcentrifuge tube before release.

Mist nets

Ten mist nets (9 m × 4 m × 36 mm mesh size) (Fig. 1) were positioned across potential flight paths of bats (trails or streams). Mist nets were set at a single transect from 19:00 and were monitored continuously until 23:00 or until it rained. A small wing punch was collected from each captured bat into a 1.5 mL ethanol-filled microcentrifuge tube following the procedures of AMNH (2013). Scissors and forceps were cleaned with alcohol and sterile tissues between bats to avoid cross-contamination. Mist nets were only used for four weeks (two nights per week) in November.

Baited hair traps

Ten hair traps (PVC pipes covered with reversed duct tape on the inside) (Fig. 1) were set per transect at 10 m intervals and monitored daily. Hair traps were baited with banana at the start of the 2-night cycle and collected at the end of the cycle. Any hair samples from traps were collected into a 1.5 mL microcentrifuge tube using sterile forceps.

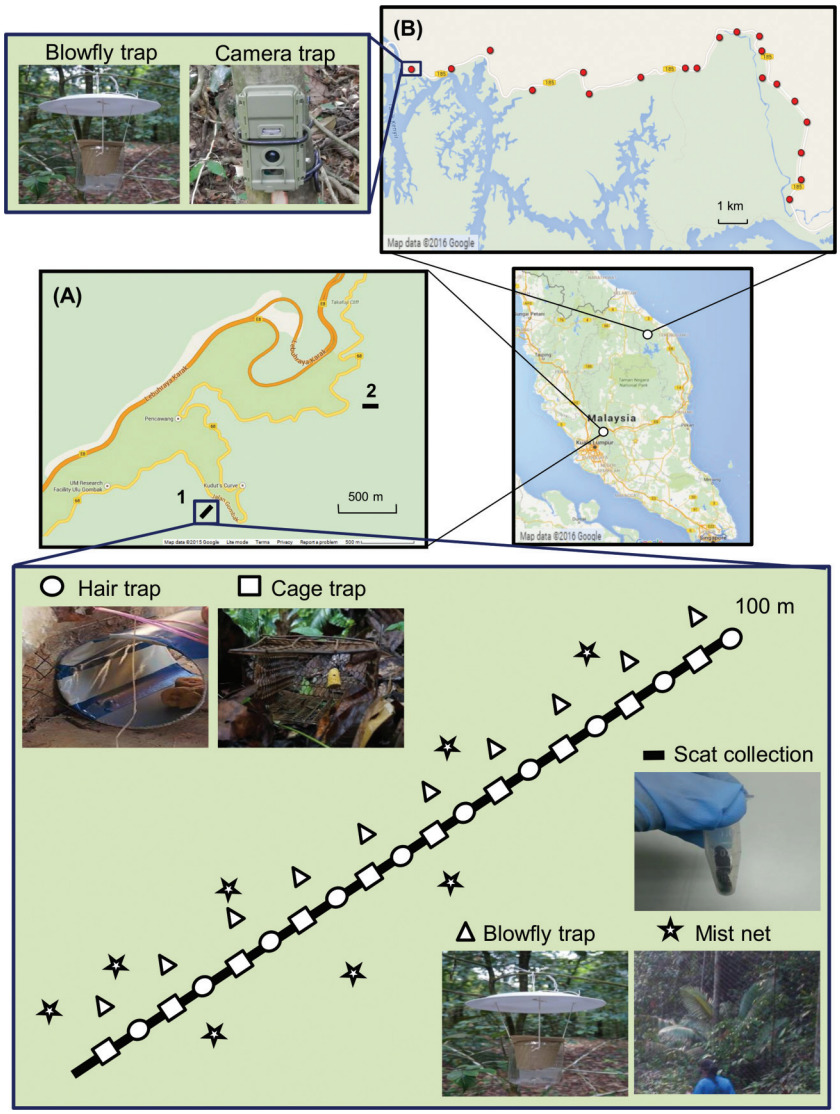
Scat collection

Any scat (Fig. 1) encountered while walking along transects daily was collected into a 1.5 mL microcentrifuge tube.

Baited blowfly traps

Ten blowfly traps baited with rotten fish (see Lee et al. 2015) were set per transect at 10 m intervals and 2 m above the ground. The traps were emptied every 24 h, and collected blowflies (Diptera: Calliphoridae) were frozen at –20 °C within 5 h of trap emptying. The guts of all collected blowflies (n = 315) were then dissected with sterile implements and pooled (five individual blowfly guts per tube) for DNA extraction (63 extractions in total). The DNA extracts were further pooled by week and transect of sampling, resulting in (8 × 2) 16 pooled DNA extracts for bulk PCR.

Fig. 1. The locations of field sites (A) Ulu Gombak Forest Reserve showing the location of two transects and the experimental design for comparing five different field methods. (B) Tembat Forest Reserve showing the locations of 20 trap stations each with a blowfly trap and a camera trap. Map data: Google.



Field methods 2

Our second field survey was conducted at TFR between 11 April 2015 and 27 May 2015 (30 days in total). A 28 km transect was used incorporating baited blowfly traps and camera traps (Fig. 1). A longer transect was used, compared to UGFR, in accordance with the longer transects generally used for camera trapping surveys (Clements 2013).

Baited blowfly traps

Twenty baited blowfly traps (Lee et al. 2015) were set along the transect (Fig. 1), 2 m above the ground, and 500 m apart. As above, the traps were emptied every 24 h, and collected blowflies were frozen at –20 °C within 5 h of trap emptying. The guts of all collected blowflies (n = 1345) were then dissected with sterile implements and pooled (five individual blowfly guts per tube) for DNA extraction (269 extractions in total). The DNA extracts were further pooled by sampling day, resulting in 30 pooled DNA extracts for bulk PCR.

Camera traps

Twenty Panthera V4 camera traps with passive infrared motion sensors were deployed along the transect (Fig. 1), attached to tree trunks approximately 50 cm above ground level. Camera traps were placed on linear features known to have high detection probabilities for large mammals (e.g., animal trail, ridge, or old logging road; Harmsen et al. 2010). No attractants were employed around the cameras. The cameras were triggered by motion, and only photocaptures that were obtained during the sampling period of the blowfly traps (30 days) were used for analysis and were catalogued using Camera Base version 1.4 software (Tobler 2012).

Species-level identification of blowfly-derived DNA

Our protocol for minimally-invasive collection of mammal DNA samples (hair, wing punches) from mammals trapped in cages or nets has been approved by the

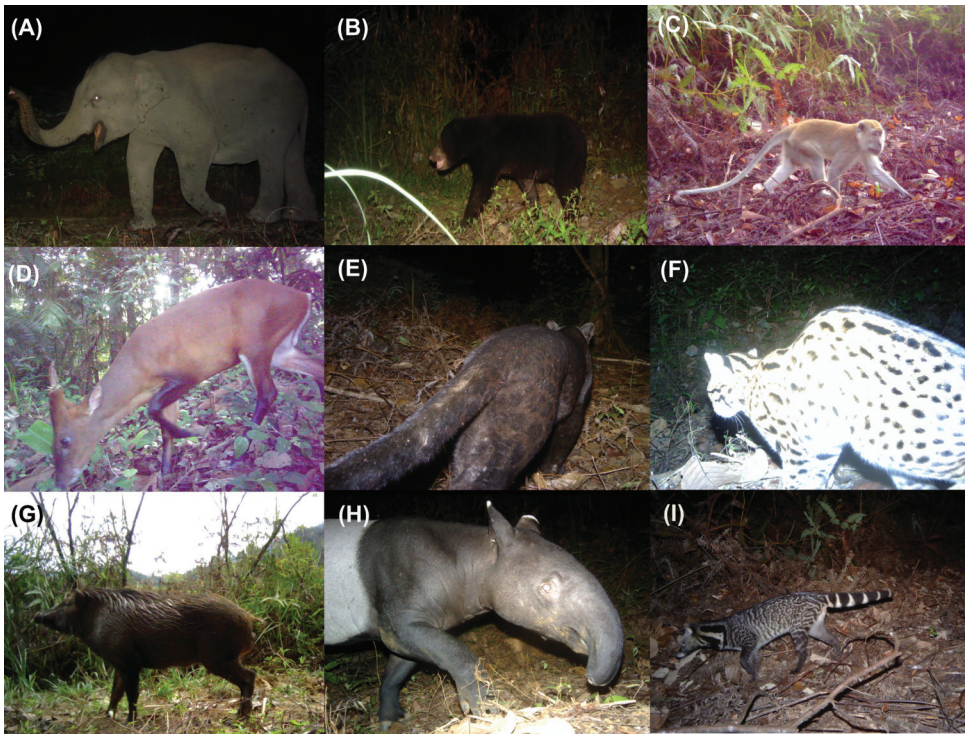
University of Malaya Institutional Animal Care and Use Committee (UMIACUC) (Ref. ISB/02/1212013/JJW (R)) and the Department of Wildlife and National Parks, Peninsular Malaysia (Ref. JPHL&TN(IP): 80-4/2 Jld16(24)). DNA was extracted from the collected hair and bat tissue samples using an alkaline lysis extraction method (100 μL elution; modified from Ivanova et al. 2012). The samples collected into 1.5 mL microcentrifuge tubes were first cut into smaller fragments using sterile scissors. 35 μL of alkaline buffer (0.1 mol/L NaOH, 0.3 mmol/L EDTA, pH 13.0) was then added into the tube, followed by centrifugation for 1 min at 110 000*g* and incubation at 95 °C for 20 min. A total of 65 μL of neutralization buffer (0.1 mol/L Tris-HCl, pH 7.0) was then added to the tubes, followed by vigorous vortexing and centrifugation (110 000*g*, 1 min). DNA was extracted from scat using a QIAamp DNA stool mini kit (QIAGEN, USA) following the manufacturer's protocol (200 μL elution). DNA was extracted from blowfly guts using a NucleoSpin Tissue kit (Macherey-Nagel, Germany) (100 μL elution) following the manufacturer's instructions.

The extracted DNA samples from hair, bat tissue, and scat samples were used for PCR in standard protocols following Wilson (2012) and Wilson et al. (2014). Primers VF1d_t1 and VR1d_t1 (Ivanova et al. 2012) were used for a first pass and RonM and VR1 for a second pass (Ivanova et al. 2012). PCR was performed using EconoTaq PLUS 2× Master Mix (Lucigen, USA) and *COI* Fast thermocycling program (see Wilson 2012), with slight modification of the amount of DNA template added (0.5–2 μL) depending on DNA extraction method. PCR products were then Sanger-sequenced by a local company (MYTACG-Kuala Lumpur, Malaysia) using the reverse PCR primer. Each sequence generated from individual specimens was trimmed of primers and edited using CodonCode Aligner (CondonCode Corp., USA) following Wilson (2012).

For blowfly-derived DNA, a 205-bp *COI* amplicon was generated using a two-step PCR that utilises universal mammal primers, Uni-Mini-bar F and RonPing R (Lee et al. 2015), with partial Illumina adaptor sequences, TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG and GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG, incorporated onto the 5′ end of the forward and reverse primers, respectively. EconoTaq PLUS 2× Master Mix (Lucigen, USA) and *COI* Fast thermocycling program (see Wilson 2012) were used to generate amplicons from 1 μL of DNA extract (five replicate PCRs were performed for each pooled DNA extract, together with a negative control). PCR products were visualised on a 2% agarose gel stained with 1× GelRed (Biotium, USA), and gels were extracted and purified using NucleoSpin Gel and PCR Clean-up kit (Macherey-Nagel, Germany) following the manufacturer's instructions. Of the 16 pooled DNA extracts from UGFR, seven produced PCR products and were selected for high-throughput (next-generation) DNA sequencing (HTS). Of the 30 pooled DNA extracts from TFR, 17 produced PCR products and were selected for HTS. The purified PCR products were used as templates for a second round of PCR reaction to generate amplicons containing Illumina adaptors and unique dual-index multiplex identifier (MID) tags. Briefly, 6 μL of the purified PCR product was mixed with 10 μL of NEBNext® High-Fidelity 2× Master Mix (New England Biolabs, USA) and 2 μL of each Nextera XT N70X and N50Y dual-index primers (Illumina, San Diego, Calif.) to make up a total of 20 μL reaction volume. The thermal cycling conditions were 98 °C for 30 s; followed by 6 cycles of 98 °C for 10 s, 65 °C for 30 s, and 72 °C for 30 s; and a final extension at 72 °C for 5 min. The PCR products were subsequently purified and size-selected using 0.8× volume ratio of AMPure XT® beads (Beckman Coulter Inc., USA). The final amplicons from all samples were quantified using KAPA Library Quantification Kit (KAPA Biosystems, CapeTown, South Africa), normalized, pooled, and then sequenced on the Illumina MiSeq Sequencer (model number: M02133; 2 × 250 bp paired-end read setting) located at the Monash University Malaysia Genomics Facility. The sequencing run produced 5 541 198 paired-end reads corresponding to 2403 megabases data output. The MiSeq outputs (FASTQ) were submitted to the NCBI Sequence Read Archive under project accession number SRP064503. The Illumina reads were demultiplexed and trimmed of adapters onboard the MiSeq using the MiSeq Reporter Software. The "raw" output of paired-end reads was quality filtered using PRINSEQ (Schmieder and Edwards 2011) by sequence length (75–350 bp), minimum mean quality score (>25), and GC content (20%–50%). Reads with low complexity (threshold using Entropy = 80) and characters other than A, C, T, or G were excluded. Redundant reads (exact sequence duplicates, 5′ sequence duplicates, 3′ sequence duplicates, reverse complement exact sequence duplicates, and reverse complement 5′/3′ sequence duplicates) were dereplicated. Reads with mean scores of less than 5 (sliding window size = 5, step size = 5) or minimum tail length of poly-A/T tails of five at 5′ end and five at 3′ end were trimmed of low-quality bases at 5′ end (1 bp) and 3′ end (1 bp). The output data (in FASTA format) was then further filtered and trimmed of primer sequences with quality control and filtering steps performed manually in CodonCode Aligner (CondonCode Corp., USA) following Brandon-Mong et al. (2015). Both consensus and singleton reads surviving the quality control and filtering steps were retained for taxonomic assignment.

The resulting DNA barcodes obtained from all individual specimens collected from cage traps, mist nets, and scat collection, and unique DNA metabarcodes from blowfly-derived DNA were uploaded to the Barcode of Life Data Systems (BOLD) (Ratnasingham and Hebert 2007) and are available in the public dataset DS-BDNGS. Linnaean species names were assigned to our DNA barcodes (individual specimens collected from cage traps, mist nets, and scat collection) and a representative DNA

Fig. 2. Photographic evidence of nine mammal species detected by camera traps at Tembat Forest Reserve, Terengganu. (A) *Elephas maximus*, (B) *Helarctos malayanus*, (C) *Macaca fascicularis*, (D) *Muntiacus muntjak*, (E) *Panthera pardus*, (F) *Prionailurus bengalensis*, (G) *Sus scrofa*, (H) *Tapirus indicus*, (I) *Viverra zibertha*. Camera settings: sample rate of passive infrared sensor = 70 times per s; flash brightness = normal (Xenon flash system); LED stealth mode = off; daylight images per trigger = 3 with delay period between images = 1 s; flash images per trigger during night time = 1 with delay period between images = 20 s; image resolution = 3 megapixel.



metabarcode (recovered from blowfly-derived DNA) when they had sequence similarity matches of >98% to DNA barcodes with Linnaean species names (submitted by other BOLD users) in the full database of BOLD (see Wilson et al. 2014). In the case of conflicts, i.e., our DNA (meta)barcodes had sequence similarity matches of >98% to database DNA barcodes with several different Linnaean species names, we assigned the Barcode Index Number (BIN) (Ratnasingham and Hebert 2013) of the matching reference DNA barcodes to our DNA (meta)barcodes without using a species epithet. When our DNA (meta)barcodes had sequence similarity matches of <98% but >94% with reference DNA barcodes, we assigned the genus name of the closest matching reference DNA barcode to our DNA (meta)barcodes (see Zeale et al. 2011). When our DNA (meta)barcodes had sequence similarity matches of <94% but >90% with reference DNA barcodes, we assigned the order name of the closest matching reference DNA barcode to our DNA (meta)barcodes (see Zeale et al. 2011). DNA (meta)barcodes sharing <90% sequence similarity to the closest matching DNA barcode in BOLD were discarded from further analyses but are discussed anecdotally below.

Species identification from camera traps

Identification of species from the photocaptures (Fig. 2) was carried out by LPS and GRC based on morphological characters with the aid of Francis (2008). Photocaptures of mammals that could not be identified with certainty due to poor lighting or blurred images were excluded.

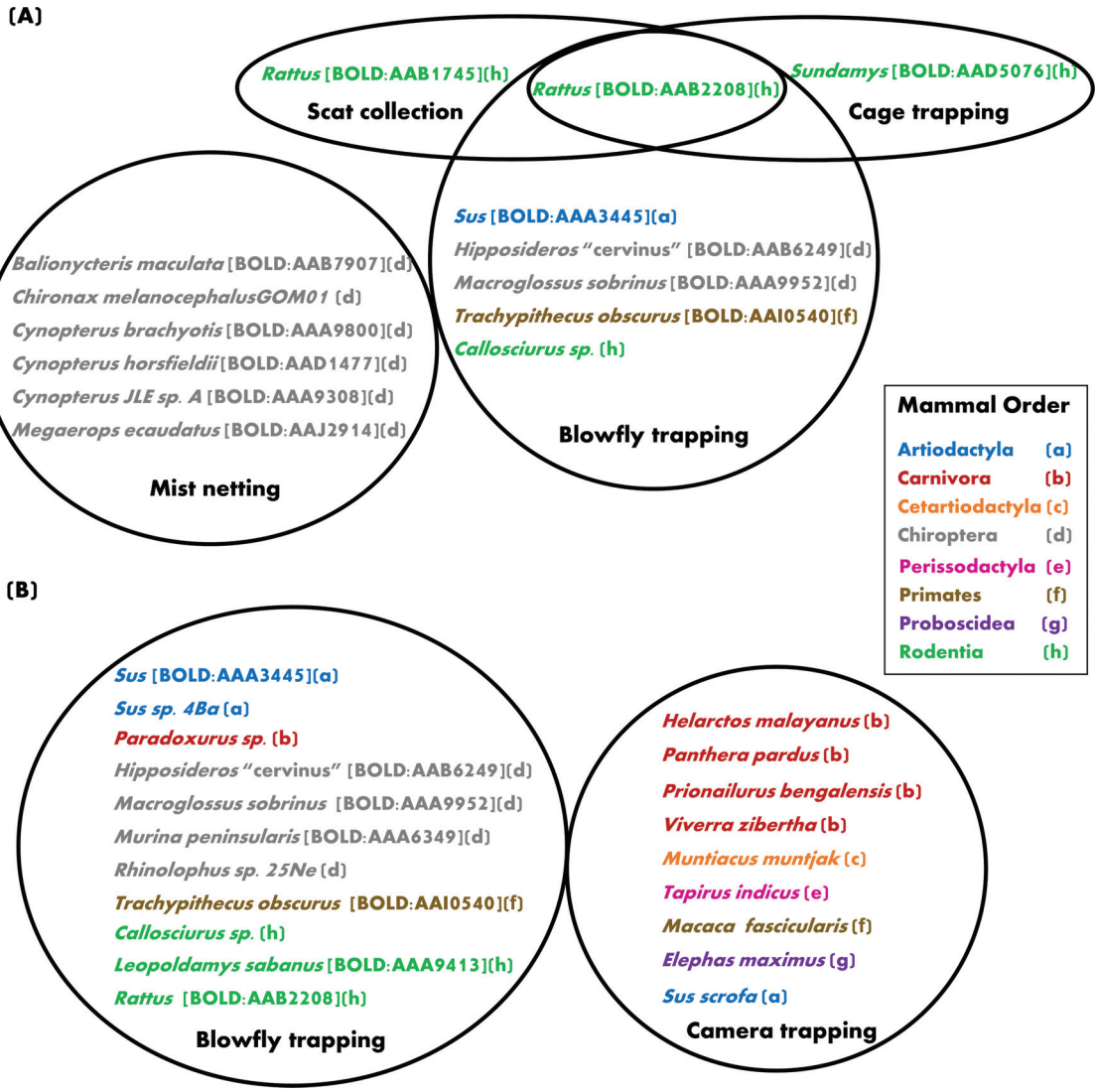
Field method calibration

For the methods used at UGFR, the percentage of potentially detectable species in the UGFR checklist (Table S1[2]) detected by each field method was calculated. The expected species richness (using Chao 1) for each field method was computed in EstimateS Version 9.1.0 (Colwell 2006). Chao 1 is suitable for species richness estimation of our sampling sites as it corrects for bias due to sampling effort and species rarity (Chao 1984; Iknayan et al. 2014). Rarefaction curves of expected species richness (using Chao 1) with 95% confidence intervals were generated based on the cumulative sampling days using EstimateS Version 9.1.0 (Colwell 2006; Fig. 4). The sampling completeness ratio (observed species richness/expected species richness; Soberón et al. 2000) was calculated for each method.

Results

At UGFR, a total of 14 mammal species from the orders Artiodactyla, Chiroptera, Primates, and Rodentia (Fig. 3) were detected from a total effort of 1440 trap days (320 from cage traps, 160 from mist nets, 320 from hair traps,

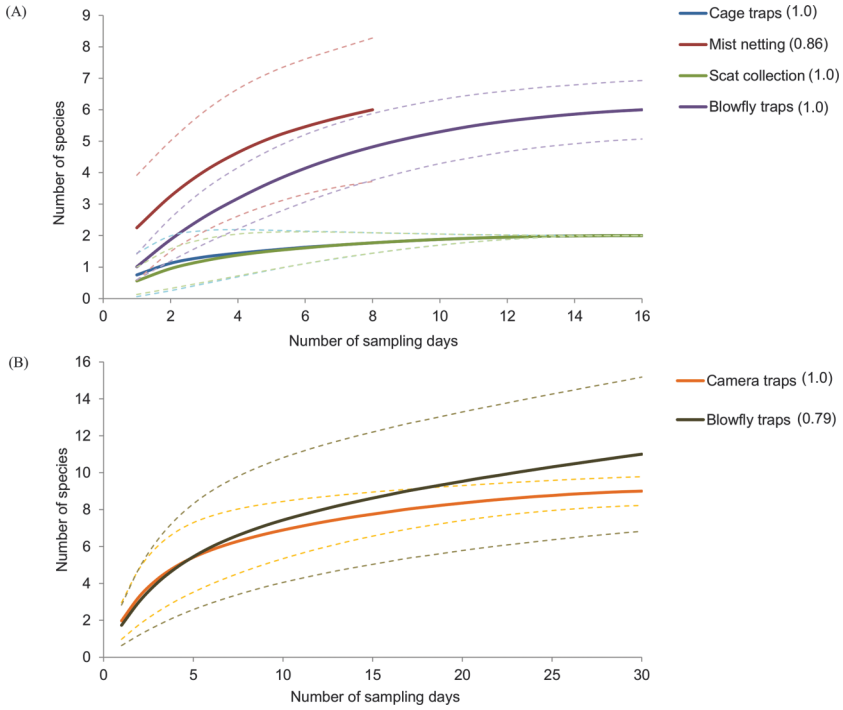
Fig. 3. Species detected by different field methods including species unique to each method and species shared across different methods at (A) Ulu Gombak Forest Reserve and (B) Tembat Forest Reserve. The number (in square parentheses) represents the BIN number assigned in BOLD to the species detected.



320 from scat collection, and 320 from blowfly traps). Mist nets and blowfly traps contributed the most species-rich samples with six species (including BINs without formal names) detected each, followed by cage traps and scat collection that detected two species each; hair traps detected no species (Fig. 3). Blowfly traps recorded species from four orders—Artiodactyla, Chiroptera, Primates, and Rodentia (Fig. 3). The other field methods detected only a single order each, except hair traps that detected no species. Two domesticated mammal species, *Bos* (under BIN BOLD:AAA2294) and *Felis* (BOLD:AAC2892), and non-mammal taxa including one bird species (*Gallus gallus*, BOLD:AAA3630), a midge species, and a fish species were also detected in blowfly-derived DNA, but these were excluded from further analyses as our focus was the detection of wild mammal species. Of 20 potentially detectable species of small-bodied rodents and treeshrews recorded in the UGFR checklist, both cage traps and blowfly traps detected two species (10.0%). Of 57 potentially detectable bats recorded in the UGFR checklist, mist nets detected six species (10.5%), and blowfly traps detected two species (3.5%). Of 45 potentially detectable non-volant mammals recorded in the UGFR checklist, blowfly traps detected the highest number of species ($n = 4$; 8.9%). Blowfly traps detected one new record of non-volant mammal for the UGFR checklist—*Trachypithecus obscurus* (BOLD:AAI0540). Sampling completeness ratios for blowfly traps, cage traps, and scat collection were 1.00, and for that of mist nets it was 0.86. The HTS data from UGFR included reads from five blowfly species—*Chrysomya* (BOLD:AAC4787, BOLD:ACD5557, BOLD:ACF0516) and *Lucilia* (BOLD:AAA6618, BOLD:ACQ1337).

At TFR, a total of 20 mammal species from the orders Artiodactyla, Carnivora, Cetartiodactyla, Chiroptera, Perissodactyla, Primates, Proboscidea, and Rodentia were detected from a total effort of 1200 trap days (600

Fig. 4. Rarefaction curves showing expected species richness (using Chao 1 with number of randomisations = 100) and sampling completeness ratios (in brackets) for each field method at (A) Ulu Gombak Forest Reserve and (B) Tembat Forest Reserve. Dashed lines show 95% confidence intervals of expected species richness for each field method at (A) Ulu Gombak Forest Reserve: dashed blue lines for cage traps, dashed red lines for mist nets, dashed green lines for scat collection, dashed purple lines for blowfly traps and (B) Tembat Forest Reserve: dashed orange lines for camera traps, dashed black lines for blowfly traps.



from camera traps and 600 from blowfly traps; see Fig. 3). Blowfly traps detected a higher number of species ($n = 11$), including the five orders Artiodactyla, Carnivora, Chiroptera, Primates, and Rodentia, than camera traps ($n = 9$), including the six orders Artiodactyla, Carnivora, Cetartiodactyla, Perissodactyla, Primates, and Proboscidea. While only one potentially domesticated mammal species was detected (*Bos*, BOLD:AAA2294), its identity could also be that of a wild bovid known from TFR (*Bos gaurus*). Non-mammal taxa, including a bird (*Gallus gallus*, BOLD:AAA3630), three fish species, a lizard species, a snake species, another squamate, and a turtle species, were also detected from blowfly-derived DNA but were excluded from further analyses. Additional non-mammal taxa detected, but which had sequence similarity matches of <90% with DNA barcodes in BOLD, included an algae (76%–80% sequence similarity) and another snake (89% sequence similarity); these were also excluded from further analyses. Sampling completeness ratio of blowfly traps was 0.79 and 1.00 for camera traps (Fig. 4). The HTS data from TFR included reads from five blowfly species—*Chrysomya* (BOLD:AAA5667, BOLD:AAB3064, BOLD:ACD5557, BOLD:ACF0516) and *Lucilia* (BOLD:ACQ1337).

Discussion

Blowfly traps and mist nets detected the highest number of wild mammal species (6 species each) at UGFR. These were followed by cage traps and scat collection, which detected two species each. Our hair traps did not detect any species at UGFR, but the difficulties associated with obtaining mammal DNA samples from hair traps have already been documented in Malaysia (see Hedges et al. 2015*b*). There was no difference in the number of non-volant small-bodied mammal species detected by blowfly traps and cage traps (2 species each). Only one species was detected by multiple field methods (cage traps, scat collection, and blowfly traps)—*Rattus* (BOLD:AAB2208). Blowfly traps were less effective than mist nets in detecting bat species (2 versus 6 of 57 in the checklist). The effectiveness of mist nets in capturing bat species, and consequently making a significant contribution to overall mammal species richness estimates, has also been shown in other studies in Peninsular Malaysia (Jayaraj et al. 2012, 2013). The number of species detected by our mist nets is similar to another study at UGFR that detected seven species using 10 mist nets and four harp traps over nine trap days (Sing et al. 2013). There was no species detected by both mist nets and blowfly traps. All species detected from cage traps and scat collection were detected at both transects at UGFR, except one species only detected from two cage traps at transect 1—*Sundamys* (BOLD:AAD5076). For blowfly traps, all species were detected at both transects except two species—*Sus* (BOLD:AAA3445), which was only detected at transect 1, and *Macroglossus sobrinus* (BOLD:AAA9952), which was only

detected at transect 2. At TFR, blowfly traps detected higher species richness than camera traps—11 species from the blowfly traps versus 9 species (based on visual identification) from camera traps. Blowfly traps detected more mammal species that were volant and arboreal in nature than those detected by camera traps. Only one species was potentially detected by both blowfly traps and camera traps—*Sus* (BOLD:AAA3445), which is probably "*Sus scrofa*" from the camera traps.

The blowfly traps at UGFR detected the highest number of orders (4 orders), in contrast to the traditional methods that detected species from only a single mammal order each. Cage traps can potentially detect small non-volant mammals of several orders but in our study only detected Rodentia, probably due to the placement of cage traps on the ground where they can be avoided by arboreal mammals (see Fontúrbel 2010). Mist nets are restricted in only being able to detect Chiroptera. Scat collection should, theoretically, not be restricted to detecting any specific mammal orders, but detection likely depends on the range size of the individuals of a given species relative to the length of transect patrolled; in our study only Rodentia were detected from scat. The number of orders detected by blowfly traps at UGFR increases (to 5 orders) when the two domestic mammal species *Bos* (BOLD:AAA2294), from Artiodactyla, and *Felis* (BOLD:AAC2892), from Carnivora, were included. The blowfly traps at TFR detected fewer orders compared to the camera traps (5 versus 6 orders). However, the comparison may be less meaningful given the lack of taxonomic equivalency (Bertrand et al. 2006) between the orders of large charismatic megafauna (specifically targeted by the camera traps) and other mammal orders. For example, Perissodactyla and Proboscidea are orders containing only a single extant species in Peninsular Malaysia. Nine vertebrate orders were detected by blowfly traps at TFR when a bird species (*G. gallus*, BOLD:AAA3630), three fish species, a lizard species, a snake species, a squamate, and a turtle species, whose DNA was also detected in the blowfly guts, were included.

The detection of a broad diversity of mammals and other vertebrate orders from blowfly traps is consistent with the study by Calvignac-Spencer et al. (2013*a*), who detected six mammal orders, an avian order, and an amphibian order from blowfly-derived DNA sampled at Taï National Park, Côte d'Ivoire, and Kirindy Forest, Madagascar. Although these results demonstrate the broad range of blowfly hosts, biases in PCR primer binding affinity may lead to sequences of certain species being amplified less efficiently than others (Deagle et al. 2014; Thomsen and Willerslev 2015). This may limit the detections to species with higher primer binding affinity, resulting in species diversity being underrepresented (Shokralla et al. 2012). Lee et al. (2015) found the primers used in this study exhibited no strong amplification bias across mammal species (from Peninsular Malaysia), but the test only included a limited number of species ($n = 41$).

One further uncertainty in the use of blowfly-derived DNA for mammal diversity assessments is potential blowfly feeding biases and dispersal distances. Surprisingly the blowfly traps did not detect *Macaca fascicularis*, the long-tailed macaque, at UGFR and TFR despite our frequent observation of numerous individuals at the site throughout the sampling period. Nevertheless, the blowfly traps at UGFR did detect a different primate—*Trachypithecus obscurus* (BOLD:AAI0540), the dusky leaf monkey, which is a near threatened species (IUCN 2015). This species is known to occur in the vicinity of UGFR, but it had not been reported from the reserve itself. It is possible, however, that the blowflies fed on *T. obscurus* tissue or faeces outside UGFR before moving into our sampling area. The few studies on blowfly dispersal, from temperate and subtropical regions (Braack and Retief 1986; Smith and Wall 1998; Tsuda et al. 2009), suggested individuals travel 100–2400 m per day, but there is no data available on blowfly travelling distances in the tropics. It is also not known whether blowflies, as opportunistic feeders (Calvignac-Spencer et al. 2013*b*), are more likely to have fed on carcasses, wounds, or faeces. All field methods recovered a low percentage of their potentially detectable species from the UGFR checklist (2%–10%), which may be an artefact of the short sampling period and restricted spatial scale of sampling relative to the total size of forest reserve, rather than feeding or primer biases. However, UGFR has more than 20 years of logging history (Nor Hashim and Ramli 2013), and megafauna recorded in 1961 (Medway 1966), such as *E. maximus*, is highly likely to be extirpated as no records of activities or populations have been published since.

Another current limitation of the blowfly-derived DNA approach is the reliance on public DNA databases to assign detected species formal species names (Collins and Cruickshank 2013). Five out of 14 wild mammal species (36%) detected at UGFR and 6 out of 20 species (30%) detected at TFR could not be assigned traditional species names. For example, *Rattus* (under BIN BOLD:AAB2208) detected from blowfly-derived DNA, cage traps, and scat collection likely represents *Rattus* "sp. R3" (Pages et al. 2010), a classic "dark taxon" (see Wilson et al. 2014) whereby it has not yet been formally described and is likely to be reported as *R. andamanensis*, *R. argentiventer*, or *R. tanezumi* in studies relying on visual diagnosis (Robins et al. 2007; Pages et al. 2010; Latinne et al. 2013; Li et al. 2015). The BIN associated with this taxon comprises DNA barcode members under the traditional names *Rattus tanezumi*, *Rattus tiomanicus*, *Rattus rattus*, *Rattus* sp., and *Rattus* sp. abtc47994 in BOLD. The two DNA barcodes named *Rattus rattus* (JF459864 and JF459865 in GenBank) had been flagged earlier as problematic and attributed to misidentification or contamination (Shen et al. 2013). In addition, *Rhinolophus* sp. 25Ne showed no species-level

match (>98%) in BOLD despite many bat DNA barcodes from Peninsular Malaysia being present in BOLD (Lim and Wilson 2015). *Sus* sp. 4Ba, detected at TFR, also showed no species-level match (>98%) in BOLD, despite the presence of 218 DNA barcodes of *Sus*, suggesting a cryptic species. We could only assign formal genus names to DNA metabarcodes detected from blowfly-derived DNA, whose closest matches were *Callosciurus notatus* (under BIN BOLD:AAF8467) (93%–94% sequence similarity) and *Paradoxurus hermaphroditus* (KC894743 in GenBank; only two sequences of ≤421 bp available in BOLD, so there is no BIN) (95%). The 205-bp DNA mini-barcode region amplified by the primers used in this study was successful in distinguishing 94% of 113 examined mammal species from Peninsular Malaysia (mined from GenBank) in a previous study by Lee et al. (2015), including separating 26 "dark" bat taxa.

The utility of blowfly-derived DNA in identifying threatened mammal species in tropical forests remains questionable. However, this is also true for other field methods (e.g., cage traps, mist nets, and scat collection used in our study) that rely on DNA-based identification for accurate assignment of formal species names (Wilson et al. 2014). As species boundaries are increasingly being refined and recognised on the basis of molecular data, especially for species-rich groups (Francis et al. 2010; Pages et al. 2010), but even for charismatic megafauna (Wilting et al. 2015), field methods that rely solely on visual diagnosis (e.g., camera traps) will have increasingly limited utility. It also remains to be seen whether blowfly-derived DNA can yield nuclear DNA of appropriate quality for individual identification (Calvignac-Spencer et al. 2013*b*). Currently, camera trapping is the most popular approach to monitor populations of mammal species, but this is restricted to species that have individuals with unique markings (e.g., spotted and striped felids; Henschel and Ray 2003; Maffei et al. 2005; Hedges et al. 2015*a*), or to individuals that can be marked prior to camera trapping (Trolle and Kery 2003; Rowcliffe et al. 2008).

At UGFR, the sampling completeness ratio for all field methods exceeded 0.86, while at TFR, the sampling completeness ratio of camera traps was 1 and of blowfly traps was 0.79. This suggested that with further sampling, blowfly traps may continue to detect a higher number of species. One reason for the high values for sampling completeness of the traditional field methods, despite low detection of the known species present, could be the limited distribution of traps over representative habitats in the study site, as some mammal species may be restricted to specific habitat types (Geier and Best 1980; Goulart et al. 2009). The dispersal of blowflies could, in theory, overcome the problem of localised trap placement as blowflies will disperse across all habitat types (Norris 1965). This could explain the detection of *T. obscurus* in blowfly traps as noted above. Alternatively, for all the field methods, except blowfly traps and potentially hair traps and scat collection, it is relatively safe to conclude that the species detected was present at the precise location of the trap during a known (short) time interval.

Deciding on the most cost-effective technique to conduct a mammal diversity assessment in tropical forests depends on many factors. For example, blowfly traps are more prone to damage but can be easily replaced at low cost (<$1 each). During our study, six blowfly traps were lost, as opposed to four cage traps ($3 each) and two camera traps ($250 each). Camera traps may be more prone to theft than blowfly traps, and can leave a significant financial dent in the project when that happens (high-end camera traps can cost up to $1000; Rovero et al. 2013), but are less prone to damage. Nonetheless, there is high initial cost involved in purchasing reagents and equipment for DNA-based detection (DNA extraction, PCR, and next generation sequencing), as well as personnel costs for analysing samples. However, species identification of bats detected from mist nets and harp traps, and rodents detected from cage traps (as exemplified by the case of *Rattus* (BOLD:AAB2208)) with an acceptable degree of accuracy also requires DNA barcoding (Wilson et al. 2014), together with the associated costs. High-throughput-sequencing can now be outsourced to private companies and (or) research institutes at increasingly competitive rates (see Brandon-Mong et al. 2015).

Conclusion

We have shown that blowfly-derived DNA is a feasible technique for mammal diversity assessments, especially when considering that a high number of species and orders were detected relative to traditional methods. Blowfly-derived DNA can potentially overcome ecological and taxonomical challenges associated with traditional methods when detecting threatened and rare or previously unrecorded species. While the sampling completeness ratios obtained from blowfly traps indicates a potential to detect more species with greater sampling effort, the lack of overlap in species detected by different field methods indicates employing multiple field methods may be the fastest way to obtain a representative account of species. Some uncertainties in the use of blowfly-derived DNA remain, such as the impact of blowfly dispersal, biases in primer binding affinity, and lack of a comprehensive and taxonomically-consistent DNA barcode reference library. With further calibration to address these uncertainties, blowfly-derived DNA may join the list of traditional field methods used to assess mammal diversity in tropical forests.

Acknowledgements

This study was supported by grants from the Nagao Natural Environment Foundation Japan to P.-S.L. and the Ministry of Higher Education Fundamental Research Grant Scheme (FP042–2014A) to J.-J.W., G.R.C., and Yong Kien Thai (University of Malaya). H.M.G. is grateful to the

Monash University Malaysia Tropical Medicine and Biology Platform for financial and infrastructure support. G.R.C. is grateful to permits from the Economic Planning Unit (Permit no: 3747), Department of Wildlife and National Parks for Project Harimau Selamanya (Rujukan kami: JHPL&TN(P)/80–4/2 Jld. 18(7)), Department of Forestry (Rujukan kami: JH/100Jld. 8(16)), and financial support from the Woodland Park Zoo-Panthera Wildcat Conservation Grant. The collaboration between the University of Malaya and Monash University Malaysia falls under an MoA. Yong Kien Thai provided advice and suggestions on an earlier version of this manuscript. Lee Yoon Hin and Tee Ah Jee provided assistance during fieldwork. Sing Kong Wah, Brandon Mong Guo Jie, and Thary Goh Gazi provided advice and suggestions during laboratory work. Lam Wai Yee, Foon Junn Kitt, and Sri Venkateswara Rao (Project Harimau Selamanya) provided advice and technical assistance during camera trapping. This project was presented at the 6th International Barcode of Life Conference as part of an Institutional Links Program (Queen Mary University of London-University of Malaya) supported by the Newton-Ungku Omar Fund.

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