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# Elucidating the diet and foraging ecology of the island flying fox (*Pteropus hypomelanus*) in Peninsular Malaysia through Illumina Next-Generation Sequencing

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There is an urgent need to identify and understand the ecosystem services provided by threatened animal species such as flying foxes. The first step towards this is to obtain comprehensive data on their diet. However, the volant and nocturnal nature of flying foxes presents a challenging situation, and conventional microhistological approaches to studying their diet can be laborious and time-consuming, and provide incomplete information. We used Illumina Next-Generation Sequencing (NGS) as a novel, non-invasive method for analysing the diet of the island flying fox (*Pteropus hypomelanus*) on Tioman Island, Peninsular Malaysia. Through NGS analysis of flying fox droppings over eight months, we identified at least 29 Operationally Taxonomic Units comprising the diet of this giant pteropodid, spanning 19 genera and 18 different plant families, including one new family not previously recorded for pteropodid diet. NGS was just as successful as conventional microhistological analysis in detecting plant taxa from droppings, but also uncovered six additional plant taxa. The island flying fox's diet appeared to be dominated by figs (*Ficus* sp.), which was the most abundant plant taxon detected in the droppings every single month. Our study has shown that NGS can add value to the conventional microhistological approach in identifying food plant species from flying fox droppings. However, accurate and detailed identification requires a comprehensive database of the relevant plant DNA, which may require collection of botanical specimens from the study site. Although this method cannot be used to quantify true abundance or proportion of plant species, nor plant parts consumed, it ultimately provides a very important first step

towards identifying plant taxa in pteropodid diet.

# Elucidating the diet and foraging ecology of the island flying fox (*Pteropus hypomelanus*) in Peninsular Malaysia through Illumina Next-Generation Sequencing

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## ABSTRACT

There is an urgent need to identify and understand the ecosystem services provided by threatened animal species such as flying foxes. The first step towards this is to obtain comprehensive data on their diet. However, the volant and nocturnal nature of flying foxes presents a challenging situation, and conventional microhistological approaches to studying their diet can be laborious and time-consuming, and provide incomplete information. We used Illumina Next-Generation Sequencing (NGS) as a novel, non-invasive method for analysing the diet of the island flying fox (*Pteropus hypomelanus*) on Tioman Island, Peninsular Malaysia. Through NGS analysis of flying fox droppings over eight months, we identified at least 29 Operationally Taxonomic Units comprising the diet of this giant pteropodid, spanning 19 genera and 18 different plant families, including one new family not previously recorded for pteropodid diet. NGS was just as successful as conventional microhistological analysis in detecting plant taxa from droppings, but also uncovered six additional plant taxa. The island flying fox's diet appeared to be dominated by figs (*Ficus* sp.), which was the most abundant plant taxon detected in the droppings every single month. Our study has shown that NGS can add value to the conventional microhistological approach in identifying food plant species from flying fox droppings. However, accurate and detailed identification requires a comprehensive database of the relevant plant DNA, which may require collection of botanical specimens from the study site. Although this method cannot be used to quantify true abundance or proportion of plant species, nor plant parts consumed, it ultimately provides a very important first step towards identifying plant taxa in pteropodid diet.

## INTRODUCTION

Understanding the contribution of animals to the functioning of rainforests has become an important issue in conservation biology. Conservation studies are now recognizing the need to collect qualitative and quantitative information on trophic relationships between animals and plants, not only to identify potential ecosystem service providers (Pompanon et al. 2012; Hibert et al. 2013), but also to inform management interventions for threatened species (Valentini et al. 2009a; Ando et al. 2013).

Bats (Order: Chiroptera) provide important ecosystem services such as insect pest suppression, pollination, and seed dispersal (Fujita and Tuttle 1991; Kunz et al. 2011). Characterising their diet is a fundamental step towards understanding their ecological roles. Due to their nocturnal and volant nature, invasive analysis (by capturing individuals) or indirect methods (by collecting droppings) have traditionally been used to study bat diets. Indeed, insect fragments found in faecal and stomach contents of insectivorous bats have facilitated the investigation of their trophic interactions (Clare 2014) and role in agricultural pest regulation (Kunz et al. 2011). Similarly, microscope analyses of pteropodid faeces have provided insights into their interactions with various plants (Bumrungsri et al. 2007), and their roles in pollination (Bumrungsri et al. 2013) and seed dispersal (Sritongchuay et al. 2014). However, studies on phytophagous bat diets to date have relied on physical identification of food plant species – either through direct observations of foraging bats, or microhistological identification of seeds, pollen, fruit fibres and leaf fragments in faeces and ejecta. The successful use of such methods relies on several important factors such as accessibility and visibility of foraging bats, as well as the availability of expert knowledge or resources such as reference collections. Another limitation of these conventional approaches is that they require physically identifiable remains to

be expelled by the bats; any plant parts that were consumed or expelled solely in liquid form will be missed out in the analysis (Pompanon et al 2012). Foraging studies of wide-ranging species such as flying foxes also require the use of expensive, hi-tech equipment such as GPS collars, which is often not feasible for all projects.

In the Old World, fruit bats such as flying foxes (Pteropodidae: *Pteropus* spp., *Acerodon* spp.; Kingston 2010) have become increasingly threatened by hunting for bushmeat and medicine (Mildenstein et al. 2016). Identifying their diet and roles as ecosystem service providers can help strengthen arguments for their protection. It will also help us understand the wider implications of large-scale flying fox extinctions, as these giant bats are known to interact with plants on a large landscape scale, performing ecological roles over vast transboundary areas (Epstein et al. 2009). Flying foxes are likely to be particularly important players in island ecosystems where they often serve as principal pollinators and seed dispersers (Cox et al. 1991), and where maintaining their numbers at high densities is necessary for the survival of plant communities (McConkey and Drake 2006, 2007; McConkey and Drake 2015). Such data are also important to understand the drivers and potential mitigation strategies for conflicts between fruit bats and humans (Aziz et al. 2015).

Whilst in-depth, comprehensive dietary/foraging studies have been conducted for certain flying fox species, particularly in Australia (e.g. Boulter et al. 2005; Williams et al. 2006), Oceania (e.g. McConkey and Drake 2006; Luskin et al. 2010), Japan (e.g. Nakamoto et al. 2007, 2009; Lee et al. 2009), South Asia (e.g. Mahmood-Ul-Hassan et al. 2010; Sudhakaran and Doss 2012), and Indian Ocean islands (e.g. Nyhagen et al. 2005; Oleksy et al. 2015), the diets of Southeast Asian species, which are some of the most threatened due to the additional threat of commercial hunting (Mildenstein et al. 2016), remains largely unknown. Indeed, apart from a

few studies in the Philippines (Reiter and Curio 2001; Mildenstein et al. 2005; Stier and Mildenstein 2005), Thailand (Weber et al. 2015), and Myanmar (Win and Mya 2015), all other dietary and foraging studies on Southeast Asian Pteropodidae have focused on the smaller pteropodids (e.g. Hodgkison et al. 2004; Fletcher et al. 2012; Bumrungsri et al. 2013; Stewart et al. 2014). This is of particular concern given that out of the 67 flying fox species listed on the IUCN Red List, almost half (30 species i.e. 45%) are actually found in Southeast Asia (IUCN 2016).

Although molecular analysis of pteropodid diets can potentially be used to overcome the obstacles outlined above, this approach has yet to be applied. Non-invasive DNA analyses of faeces have already been conducted to determine the herbivorous diets of animals such as primates (Bradley et al. 2007), marmots, bears, capercaillies, grasshoppers, molluscs, slugs (Valentini et al. 2009a), pigeons (Ando et al. 2013) and tapirs (Hibert et al. 2013), but this has never before been attempted for pteropodids or plant-based mammal diets in the Palaeotropics. To date however, molecular analyses of bat diets have only been used for insectivorous species (e.g. Clare et al. 2009; Razgour et al. 2011; Zeale et al. 2011). In fact, to our knowledge, the only successful attempt to identify the diet of plant-visiting bats through molecular analysis has been done by one study in the Neotropics (Hayward 2013).

On Tioman Island in Peninsular Malaysia, we evaluated the utility of Next-Generation Sequencing (NGS) to identify plant species present in the droppings of the island flying fox (*Pteropus hypomelanus*), whose diet hitherto remains unknown throughout its entire range. Specifically, our study aimed to: 1) determine the feasibility of extracting amplifiable plant DNA from flying fox droppings; 2) infer spatio-temporal dietary patterns based on high throughput amplicon sequencing of the partial *rbcL* gene; and 3) evaluate the potential of NGS

analysis in complementing or even replacing conventional microhistological analysis to elucidate flying fox diet.

## MATERIALS AND METHODS

### *Study species*

The island flying fox (*Pteropus hypomelanus*), also known as the variable flying fox and the small flying fox, roosts gregariously, forming colonies of up to 5000 individuals. It is a widespread insular species, considered to be abundant throughout a distribution range that extends from the Maldives and Indian islands in the west to Melanesia in the east. Because of this, it is considered to be Least Concern on a global scale by the IUCN Red List; however its population trend is noted to be decreasing (Francis et al. 2008; Olival 2008).

In Malaysia this species is confined to small offshore islands. A study on *Pteropus* population genetics and phylogeography (Olival 2008) has shown the east coast populations off the Malay Peninsula to be a subspecies – *P. hypomelanus lepidus* – that is genetically distinct from the west coast populations of *P. hypomelanus robinsoni.* The species is listed as Endangered on the Malaysian Red List (DWNP 2010).

On Tioman, the island flying fox can be found roosting permanently in two villages: Tekek, on the west coast, and Juara, on the east coast (Figure 1A), and forages throughout the island (Medway 1966; Ong 2000). Monthly roost counts conducted during March-October 2015 yielded estimated ranges of 675-1033 individuals in Juara, and 2178-5385 individuals for the entire island.

### *Study site*

We conducted this study on Tioman Island (2°48′38″ N, 104°10′38″ E; 136 km$^2$; Figure 1A), located 32 km off the east coast of Peninsular Malaysia in the State of Pahang. This research was approved by the Economic Planning Unit of Malaysia (Permit number: 3242). Much of the island inland is still covered by primary tropical rainforest, which has been designated as Pulau Tioman Wildlife Reserve (82.96 km$^2$). It has a hilly topography, with flat areas only along the coast (Abdul 1999). The area designated as a wildlife reserve is composed of lowland mixed dipterocarp forest and hill dipterocarp forest. Most forested areas are still inaccessible due to the rugged topography, with many steep slopes and rocky outcrops (Latiff et al. 1999). The climate is tropical, uniformly warm and humid throughout the year (Hasan Basyri et al. 2001), but the island experiences the northeast monsoon from November to March (Bullock and Medway 1966).

There are currently seven villages on the island, situated along the coastline (Fig. 1A). The majority of the local people are Muslim, and therefore due to religious dietary restrictions do not hunt the bats for food or medicine (Aziz et al. submitted). As the island's marine area is also a designated Marine Park and a popular tourist destination, many of the local people are heavily involved in the tourism industry (Abdul 1999).

Currently, the island flying fox can only be found roosting in two villages: Tekek and Juara. Local people have reported that the flying foxes do forage in other villages on the island. Besides flying foxes, only four other pteropodid species have been recorded on the island (Lim et al. 1999).

***Study design***

First, we assessed the feasibility of extracting plant DNA from *Pteropus* droppings, and evaluated whether DNA sequences obtained from NGS could be matched with those from: 1) online DNA reference databases; and 2) an *in situ* reference collection created by sampling DNA from possible food plants in and around both villages. Next, we compared the performance of NGS with a conventional microscope approach to identify food plant species from flying fox droppings.

***Sampling of flying fox droppings***

Collection of droppings took place once a month during March-October 2016 (i.e. eight months). Samples of flying fox droppings consisting of faeces and ejecta were collected for three mornings in the last week of each month from three separate day roosts in Juara (east coast) and two separate day roosts in Tekek (west coast). The number of roosts and sampling days were determined based on species accumulation curves of pollen morphospecies that were detected through preliminary microhistological analysis in June 2014. Program EstimateS (version 9.1.0; http://viceroy.eeb.uconn.edu/estimates/) indicated that sampling completeness (i.e. observed/estimated number of species; Soberon et al. 2000) was around 97% using this sampling regime.

In Juara, three suitable roost trees (Fig. 1B, right) for sampling were selected based on accessibility and also on the highest/largest amount of faecal/ejecta splatter produced under the roost, in order to maximise sample yield. As flying foxes often shifted roosts or temporarily abandoned degraded roosts, this meant that sometimes different roosts were sampled in each location every month or even every morning, although most roosts were consistently sampled each month due to their constant high occupancy and best accessibility yielding the most amount

of droppings every month. In Tekek, two suitable roosts, one mango and one jackfruit roost, were selected based on least human activity/disturbance, although this was consistently high for all accessible roosts at that site. However, after the first six months, the jackfruit roost was chopped down by the owner. Consequently the angsana roost, with higher human disturbance, was sampled as a replacement for the remaining two months at that site (Fig. 1B, left).

Plastic sheets measuring 0.8 x 1.0 m were placed under each roost after dark, once the bats had exited the roost to forage. The roosts were then visited the next morning for collection starting at 0700h and ending at 1200h (bats typically returned to the roosts around 0500-0600h); the plastic sheets were pulled out first from under the roost, and carefully moved away to a clear area for processing (Fig. 1C). As it was often difficult to differentiate faeces from ejecta (chewed-up plant parts spat out by bats during feeding), both were collected and analysed equally as 'droppings' (Fig. 1D). Droppings collected for processing were selected based on unique colour and texture, as this was assumed to be representative of plant diversity in the bats' diet. Following the approach used by Stier and Mildenstein (2005) based on short gut-passage time for flying foxes (12-34 min; Tedman and Hall 1985), we assumed that each bat voided its last meal once, and therefore each dropping represented a different individual's food choice. Droppings were collected by swabbing them with a cotton bud, then placing each individual dropping into a 5 ml Eppendorf tube containing ~1000 µl of 95% ethanol. These tubes were then kept cool in the field, either by storing in a conventional freezer or by using a portable cooler box with ice packs, for 1-3 days before being transported off the island and then stored in a -80°C freezer.

In order to simultaneously test the utility of NGS and compare it with conventional approaches, we collected two duplicate sets of 10 individual droppings from one single roost in

Juara village during a single morning on 6 May 2015. One sample set was then kept in a conventional fridge for microscope analysis, whilst the other set was stored in the -80°C freezer for molecular analysis.

***Reference plant sample collection and generation of in situ rbcL sequence database***

In order to form an *in situ* DNA reference collection, we first checked a published list of genera of known food plants for *Pteropus* across its range (Marshall 1985), cross-checked this against a preliminary checklist of seed plants for Tioman (Latiff et al. 1999), and also obtained information on possible flying fox food plants through talking to local people in Juara. We then searched for genera of similar plants in and around the two villages with the aid of a local plant expert. The botanical identification of plants (at least to genus) were subsequently verified by a trained botanist familiar with plants from the region. When the individual of a plant matching the genera was opportunistically found, we recorded its GPS location and collected 3-5 mature leaves for DNA extraction. The leaves were stored in Ziploc bags with silica gel under cool conditions to retard decomposition rates. Leaf samples from 19 different plant species were obtained for this purpose, constituting a preliminary library (Table 1).

Genomic DNA was extracted from approximately 25 mg of one leaf from each plant species using DNAeasy Plant Mini Kit (Qiagen, Halden, Germany) according to the manufacturer's protocols. DNA amplifications were performed in a mastermix containing 1µL of DNA, 25µL of OneTaq Quick-Load 2X Master Mix with Standard Buffer, (New England Biolab, Ipswich, MA), 1µL of 10mM forward primer rbcLaf-M13, 1µL of 10mM reverse primer rbcLa-revM13 (Table 2), and 22µL of nuclease-free water. The PCR protocol was started with an initial denaturation step for 30 sec at 94°C, followed by 30 cycles of 30 sec at 94°C, 30 sec at

48°C, 40sec at 68°C, and final elongation for 2 minutes at 68°C. The PCR products were purified using 0.8X volume ratio of Agencourt Ampure XP beads (Beckman Coulter, Inc). The purified samples were sent to 1st BASE SB for Sanger sequencing. The sequencing results were quality trimmed using CodonCode TraceViewer (http://www.codoncode.com/TraceViewer/) and aligned using MAFFT version 7.0 (Katoh and Standley 2013).

***Next-generation sequencing***

Individual droppings were pooled according to roost (n=5, 2 in Tekek and 3 in Juara) and month (n=8), creating 40 separate mixtures for analysis. The tubes containing the daily samples were first vortexed for 2 min to homogenise the content and subsequently, 1000 µL of the sample was pipetted into another tube to form the mixture. Next, 100 µL of the mixture underwent gDNA extraction with DNAeasy Plant Mini Kit (Qiagen, Halden, Germany) according to the manufacturer's protocols. Based on the alignment, primers targeting 220bp of *rbcL* gene were designed using Primer3 (http://bioinfo.ut.ee/primer3-0.4.0/) on default settings (Fig. 2). Partial Illumina adapter sequences were added to the 5' end of the designed primers, rbcL-357F and rbcL-556R, to allow barcoding and sequencing on the Illumina platform. The current *rbcL* was not used for Illumina as the digested plant might be degraded; hence a shorter target region would be more optimal to investigate the diet of flying foxes (Pompanon et al. 2012).

PCR reaction was performed using IlluM_rbcLF and IlluM_rbcLR. The 20µL PCR cocktail consists of 10µL _*Q*5 Hot Start High-Fidelity 2X *Master Mix* (New England Biolab, Ipswich, MA), 1µL each of 10µM forward and reverse primer, 1µL gDNA and 7µL Milliq water. All reactions were performed in a Veriti® 96-Well Fast Thermal Cycler with the following protocol: initial denaturation for 30 sec at 98ºC, 25 cycles of 10 sec at 98ºC, 30 sec at

55ºC and 10 sec at 65ºC, with a final 1 min extension at 65ºC. The PCR product was purified using 0.8x vol. ratio Agencourt Ampure XP beads (Beckman Coulter, Inc). Then, 1uL of Index 1 and Index 2 primers from Nextera XT kit were added to 3uL of purified PCR product and combined with 5uL of *Q5* Hot Start High-Fidelity 2X *Master Mix* (New England Biolabs, Ipswich, MA). The PCR protocol was as followed: initial denaturation for 30 sec at 98ºC, 25 cycles of 10 sec at 98ºC and 1 min at 65ºC, with a final 1 min extension at 65ºC.

The purified amplicons containing the full length Illumina adapter and appropriate unique barcode were then quantified using KAPA Library Quantification kit (Kapa Biosystems, CapeTown, South Africa) on the EcoRealTime PCR system (Illumina, San Diego, CA). Based on the qPCR data, the amplicons were normalised, pooled and subsequently sequenced on the MiSeq (2 x 250 bp paired-end run) located at the Monash University Malaysia Genomics Facility.

***Adapter trimming, OTU clustering and abundance estimation***

Illumina nextera adapters and the primer sequences of the reads were trimmed off using Trimmomatic v 0.33 and FastX trimmer, respectively (Bolger et al. 2014; http://hannonlab.cshl.edu/fastx_toolkit/). The trimmed paired-end reads were then merged using PEAR (Zhang et al. 2014) using default settings. Dereplication, singleton removal and Operationally Taxonomic Unit (OTU) clustering were performed using the pipeline implemented in UPARSE (Edgar 2013). The filtered OTUs were manually inspected and those containing stop codon(s) in the open reading frame were removed from the final dataset. Subsequently, to generate relative abundance distribution for each sample, reads were mapped to the final OTUs via USEARCH and normalised to 10,000 reads (Caporaso et al. 2010).

The number of reads for each OTU was converted into percentage and OTU with relative abundance lower than 0.5% was eliminated. The remaining OTUs were searched against BOLD database and reference sequences obtained from this study to obtain the identity of the OTUs. The top non-redundant 10 BLAST hits for each OTU along with the generated *rbcL* sequences from this study were used for phylogenetic construction. Briefly, the sequences were combined and aligned using default settings in MAFFT version 7.0 (Katoh and Standley 2013). The aligned sequences were subsequently used to construct a maximum likelihood phylogenetic tree using FastTree (-nt -gtr) (Price et al. 2009). Subsequent tree visualisation and editing were done using FigTree (http://tree.bio.ed.ac.uk/software/figtree).

***Microhistological analysis***

For the 10 dropping samples collected in May, we sent one set for NGS analysis (following the protocol above) and used another set for microscope analysis. First, we manually broke up the dropping contents in the tube to produce a relatively more representative liquid sample. 1-3 drops of this liquid was then dropped onto a microscope slide using a pipette. Fuchsin jelly was added to this in order to stain pollen grains within the dropping, a slip cover was placed on top, and the jelly was then melted over an open flame, sealing the slip cover to the slide. The slide was then cooled down in a conventional fridge in order to allow the jelly to solidify again before examination.

Once the slide had cooled sufficiently, it was placed under a conventional light microscope (Leica DM E) and first examined using 10/0.25 magnification in order to detect pollen grains and other plant parts. Once pollen or other plant parts were detected these were compared with a preliminary reference collection of pollen and fig parts taken from plants at the

298 sampling site (photographed using a microscope eye-piece camera (Dino-Eye AM4023X)), as
299 well as photos from Start (1974), S. Bumrungsri (unpublished) and Mohamed (2014). If
300 necessary, smaller pollen grains were viewed in greater detail using 40/0.65 magnification.
301 Since any attempt to quantify abundance of pollen grains can bias the analysis towards
302 plant species that naturally produce greater amounts of pollen than others, pollen species were
303 assessed based on 'presence/absence' only; following the advice and approach reported by
304 Thomas (2009), a plant species was considered present in the diet if three or more of its pollen
305 grains were found on one single slide.
306
307 **RESULTS**
308 ***NGS as a viable tool to study pteropodid diet***
309 We were able to successfully extract, amplify, and subsequently identify plant DNA from all of
310 the collected flying fox droppings using the *rbcL* primer. This indicates that the integrity of plant
311 DNA was not severely affected during food digestion in the flying fox gut. After the filtration, 29
312 OTUs were recovered from the sequencing reads, nominally representing at least 19 different
313 plant genera from 18 families detected in the droppings (Fig. 3, Table 3). In addition, the family
314 Polygalaceae represents a new record for pteropodid diet. Fig. 4 shows the maximum likelihood
315 phylogenetic tree that was constructed from these results. Based on sampling completeness
316 (calculated using EstimateS) for OTU relative abundance data from five roosts (data pooled over
317 three days) per month using Chao 1 species richness estimator (good for datasets skewed towards
318 low abundance classes; Chao 1984), sampling completeness was relatively high for the months
319 March, April, August, September and October (88-100%). However, sampling completeness was
320 relatively low for May, June and July (55-79%).

***Spatio-temporal dietary patterns***

The results from our NGS analysis of island flying fox droppings over eight months suggest that the diet at both Juara and Tekek during this time was dominated by four different plant taxa that each yielded more than 100 sequencing reads: *Ficus* sp. (OTU 1), *Mangifera indica* (OTU 3), *Pavetta* sp. (OTU 4); and *Uncaria* sp. (OTU 5). Spatio-temporal trends in the relative abundance of these four taxa in the diet were apparent (Fig. 5). For example, OTU 5 appeared to be consumed in similar proportions at both Juara and Tekek across all months whereas OTU 4 was consistently consumed in low proportions in Tekek yet consumed irregularly in Juara over the same period. Even between different roosts in the same site, spatio-temporal differences were observed, such as for OTU 7 (*Antiaris* sp.; Fig. 6), although this taxon was far less abundant in the diet (50 sequencing reads).

***Microhistological vs. NGS approach***

Microscope analysis identified two plant taxa in flying fox droppings (Table 4). Out of 10 individual droppings, three contained durian (*Durio* sp.) pollen. Two of these also contained fig parts (*Ficus* spp.). All the other droppings contained fig parts exclusively; no other plant parts were detected. Durian pollen occurred at extremely low abundance; in all cases, only 3-4 grains were detected per slide. No other pollen or plant parts were detected. On the other hand, NGS identified the same two plant taxa detected by microhistological analysis, and further identified an additional six plant taxa. *Durio* was not detected in the same samples as in those identified via microscope.

Using NGS for the same 10 individual samples, reads mapping to OTU 1 belonging to the genus *Ficus* were highly abundant across a majority of the samples. Only three samples

345 contained a small number of reads (<1%) mapping to OTU 17 belonging to the genus *Durio*,
346 which to some extent correlates with the observation from microhistological analysis. The ability
347 of NGS to identify at least six additional plant taxa with substantial relative abundance, that were
348 completely missed by the conventional approach, underscores its potential in uncovering plant
349 taxa previously not known to be part of flying fox diet. It is also worth noting that the relative
350 abundance of mapped reads varied considerably among individual samples which may be an
351 indication of inter- and/or intra-sample diet inconsistency.
352
353 **DISCUSSION**
354 Our study is the first to describe the diet of the island flying fox, which was previously unknown.
355 To our knowledge, this is also the first known use of NGS to identify plant taxa in the diet of a
356 pteropodid, which has been difficult to characterise due to this animal's volant nature, large
357 home ranges and nocturnal foraging behaviour. Furthermore, NGS provided comparatively
358 greater insights into its diet than conventional microhistological approaches by detecting a wider
359 range of plants, thus highlighting the comprehensiveness and discriminatory potential of the
360 newly designed *rbcL* primers. Through NGS, we also discovered a new food plant family
361 previously unrecorded by other studies of pteropodid diet. In our study, attempts to use
362 microscope analysis to identify plant parts in droppings proved to be challenging, as no pre-
363 existing reference collection was available. Attempting to build our own comprehensive
364 botanical reference collection for Tioman was time-consuming and labour-intensive – and the
365 resulting collection often did not match up with the plant parts found in the flying fox droppings.
366 Obtaining DNA from botanical specimens, however, is still a necessary step to narrow down the
367 identity of OTUs obtained from NGS to species level. More importantly, the use of NGS allowed

368 us to identify plant species even when no physical plant parts were found in the flying fox
369 droppings. Fourteen of the probable plant genera detected have also been recorded by botanists
370 as being present on Tioman, including the top four genera detected most abundantly in the
371 droppings (Latiff et al. 1999; Mohd. Norfaizal et al. 2014).

372 In order to be conservative, we have avoided assigning most OTUs in our study to species
373 level. The only exception is OTU 3, which we identified as *Mangifera indica* based on 100%
374 matches between the OTU, BOLD database sequence and botanical specimen sequence.
375 Although plant identification based on DNA sequence to the species level may not be
376 straightforward, the utilisation of partial *rbcL* gene fragments coupled with alternative taxonomic
377 assignment based on phylogenetic tree clustering has already delivered numerous new insights
378 into flying fox diet, and overcome severe limitations associated with traditional methods. It is
379 also worth noting that identification to family level is highly accurate based on the partial
380 sequence of *rbcL*, a protein coding gene associated with the chloroplast genome of all living
381 plants. We have also demonstrated that most OTUs in our study could also be successfully
382 assigned to genus level using this approach.

383 Other studies have successfully used different genetic approaches to identify plant species in
384 animal diets. Valentini et al. (2009a) found *trn*L to be effective for Asian mammals, birds, and
385 invertebrates, identifying 50% of the plant taxa found in the diets of these animals to species
386 level. The same approach has been used for European bison (Kowalczyk et al. 2011), alpine
387 chamois (Raye′ et al. 2011), and red-headed wood pigeons (Ando et al. 2013). However, it has
388 not been recommended to use a single DNA region for barcoding plants (Clare 2014). Indeed, a
389 combination of target regions has been used to study the diets of large herbivores (e.g. *rbcL* with

*ITS-2* for African primates; Bradley et al. 2007, and *trn*L with ITS1 for lowland tapirs; Hibert et al. 2013).

In our study, only six OTUs had 100% matches to the sequences of botanical specimens collected from the study site, suggesting insufficient plant sampling. It is worth noting that subsequent similarity searches against the BOLD database did not recover reference sequences with 100% identity matches for all of the OTUs. This may be attributed to gaps in the database i.e. certain plant species consumed by the flying foxes may not yet have their corresponding sequences deposited in the database. This highlights the importance of building a comprehensive local sequence library beforehand, preferably specific to one's particular study site. In addition, there is also an urgent need for the BOLD database to have more representation of plant sequences from Southeast Asia and, more specifically, from Peninsular Malaysia.

We acknowledge that NGS approaches to diet identification are semi-quantitative because chloroplast abundance is known to be variable in different plant species and different parts of the leaf. Ultimately, the ability of NGS to accurately identify food plants will always depend on sequence specificity of the primers. While the NGS approach has proven to be useful in elucidating the island flying fox's varied diet on Tioman Island, for animals with such a highly diversified phytophagous diet, primer specificity will always be a limiting factor and there is a chance that unknown plant species will not be detected due to primer mispriming. Also, identical chloroplast DNA sequences can be present in different but related species, making it impossible to distinguish closely related plant species from each other in the diet. This could be one possible factor as to why OTU 12 had 100% identity hits with several members of family Arecaceae, making it impossible to identify this OTU to genus level, and suggesting that this particular family requires further phylogenetic investigation.

Another limitation of the NGS approach for generalist diets is that it does not identify which part of the plant was consumed. For animals that are specialised frugivores and nectarivores, or large terrestrial herbivores that consume entire plants whole, this may not be an issue. Flying foxes, however, are generalists which consume fruits, flowers, nectar, and even leaves (Marshall 1985). It is this dietary plasticity which allows them to perform more than one ecological role in tropical landscapes. Therefore, identifying which plant parts are actually consumed is a crucial step towards identifying the ecosystem services that these bats provide. Because of this, NGS can only provide a first step towards identifying flying fox diet, and should not be viewed as a replacement for microhistological analysis. Nevertheless, this approach has shed new light on flying fox diet by discovering new plant species that were entirely missed out by the conventional approach. Ideally, studies using NGS should be combined with micro-histological analysis in order to fill in the gaps and broaden our understanding of pteropodid diet and foraging ecology. NGS can also be used in combination with comprehensive and long-term data on plant phenology, to observe which food resources are available at which time. Following on from this preliminary study, the identification of specific food plants via NGS can now help guide more in-depth plant sample collection and phenological observations.

NGS did not detect *Durio* equally in the same individual droppings as those identified via microscope. This is likely due to the low abundance of this plant taxon in the droppings affecting detection probability, especially since the NGS analysis used a more general primer that was not specific to *Durio.* This pollen detection probability is another caveat to be aware of; Scanlon and Petit (2013) have cautioned that faecal subsampling methods can potentially lead to inaccurate detection of pollen in dietary studies, regardless of which method is used. The sample collection method in the field, selecting only for droppings with unique colour and texture, may have also

introduced a bias that could result in underestimating the proportion of a plant taxa in the diet. In particular, sampling completeness for the months May, June and July were relatively low, showing that more roosts and/or days needed to be sampled in order to obtain a complete representation of diet for these months. Interestingly, this also suggests that diet diversity, and potentially food resource diversity, were relatively higher during these three months compared to the rest of the year. Clearly, our method of collecting only droppings of unique appearance was not sufficient to reflect the full diversity of the diet; future studies should aim to collect all droppings found underneath a roost.

It is important to note that even with the potential underestimation, figs consistently formed the highest amount of plant taxa detected in the droppings each month, at both sampling sites. This strongly suggests that figs compose the core diet of flying foxes on the island. Although our study specifies only one (unidentified) species of fig, this is a conservative estimate. Sequencing a longer fragment of the *rbcL* gene would give better resolution, indicating whether more than one fig species was consumed. It is thus highly likely that the island flying fox plays a key role in dispersing fig seeds throughout Tioman, making these bats important keystone species for the island (Cox et al. 1991; McConkey and Drake 2015); future studies on seed dispersal and germination are required to confirm this.

Given the potentially short gut passage times involved (Tedman and Hall 1985), droppings collected from day roosts in the morning may bias the analysis results towards food items that were consumed only at the end of the foraging period (Schmelitschek et al. 2009). Although Banack and Grant (2003) have observed flying foxes returning to food resources that were foraged upon earlier, before then returning to day roosts, this is still a potential caveat to bear in mind; food plants that were only consumed during the start or middle of the evening may

not have been detected by our methods. For example, primates are known to exhibit temporal patterning in diet choice, structuring their diet throughout their foraging period with different food items; it is believed that this is due to how different foods are processed, and give energy, at different rates, and therefore helps to ensure that the animals maintain high energy levels (Robinson 1984; Ganzhorn and Wright 1994; Chapman and Chapman 1991). Given the sheer size of Tioman, and the logistical challenges of observing flying foxes foraging throughout the entire evening, the only way to overcome this possible information gap is to conduct GPS tracking studies.

## CONCLUSION

Our study is the first to use NGS to identify potential plant species in flying fox diet, paving the way for a new approach to studying pteropodid diets. Since our NGS analysis of flying fox diet was semi-quantitative, it is not yet possible to make any definite conclusions regarding food preference vs. food availability; ultimately it is unclear to what extent sampling bias and detection probability may have influenced the type and abundance of plant taxa detected in our study. Yet some of the interesting patterns we observed are worth investigating in greater detail, particularly in combination with microhistological analysis. The results will also help to guide us in conducting more accurate and expanded phenology monitoring, and further collection of botanical samples. Further and more rigorous sampling, especially at the level of the individual animal, is required to understand the dietary patterns of this particular flying fox population, expand on the information provided here and build on our understanding of how the island flying fox may act as a strong interactor within the ecosystem of Tioman Island.

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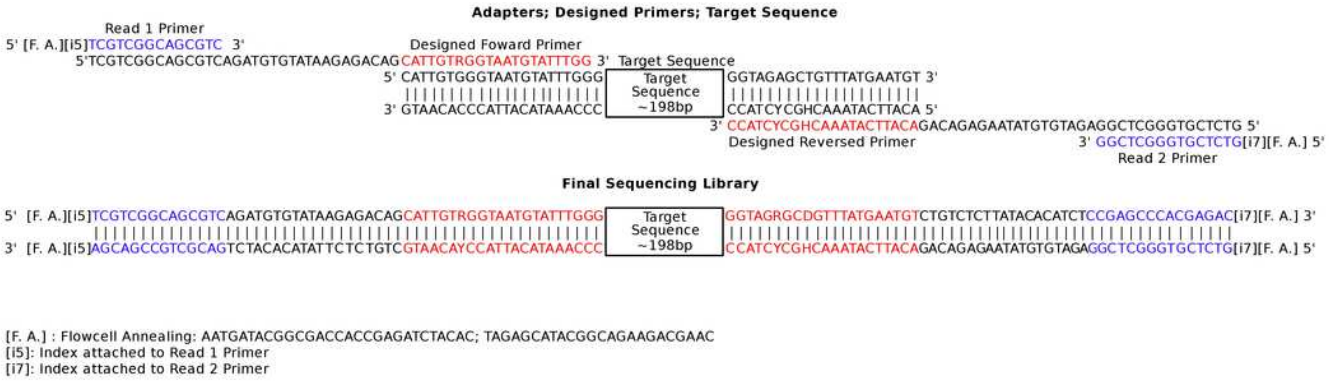
# Figure 1

Map of study area and images of sampling site and method.

A) Map of Tioman showing sampling sites Tekek & Juara. B) Examples of flying fox roosts sampled in Tekek (left) & Juara (right). C) Collecting droppings from roosts. D) Close-up of droppings.

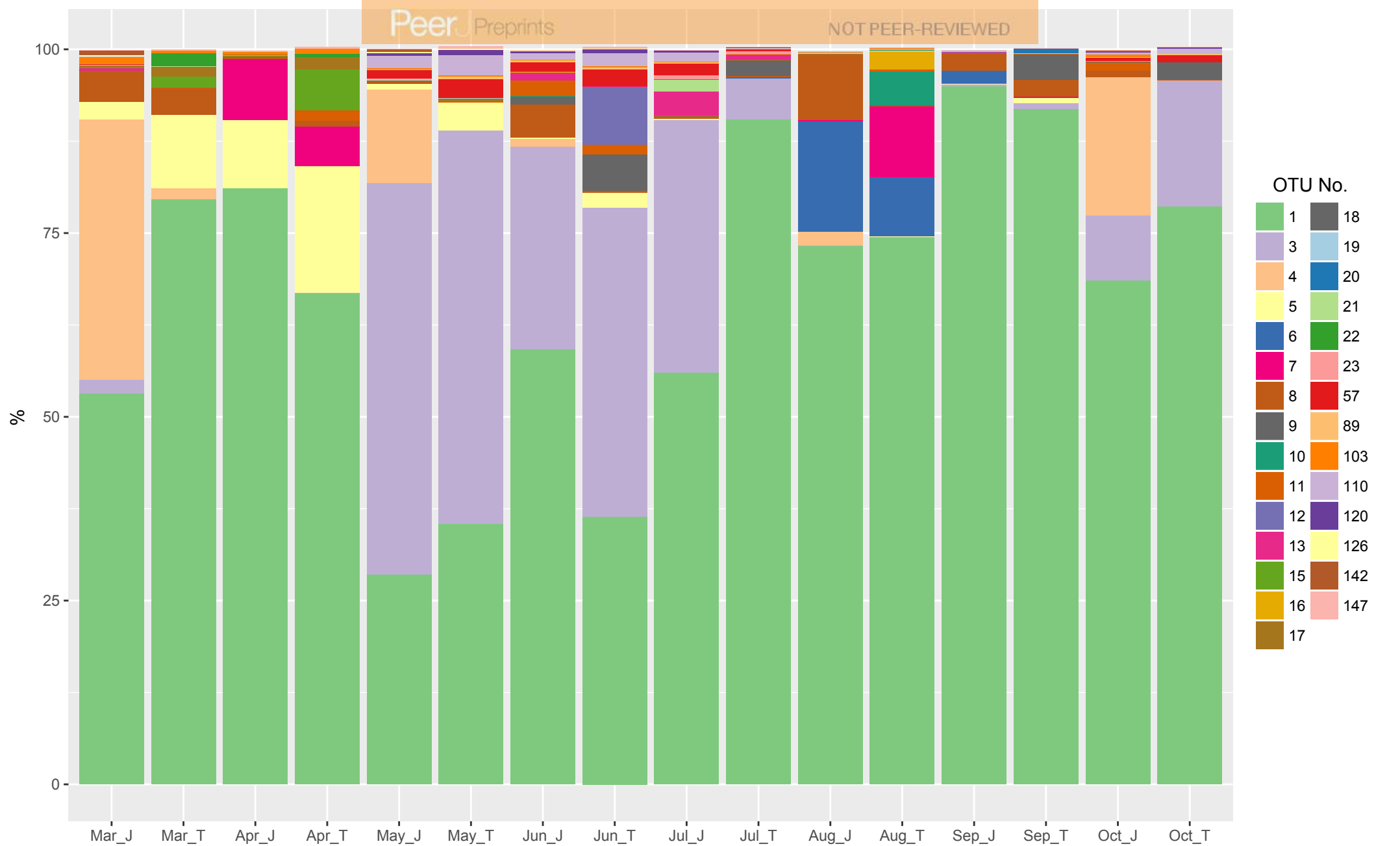
# Figure 2

Overview of the newly designed primers and expected construct consisting of the complete Illumina adapter, dual index barcode and partial *rbcL* gene.



## Figure 3(on next page)

Proportion of OTU reads detected in flying fox droppings across 8 months (Mar-Oct 2015) at 2 different roosting sites on Tioman, Juara (J) and Tekek (T). Refer to Table 3 for OTU identification and corresponding number of reads.

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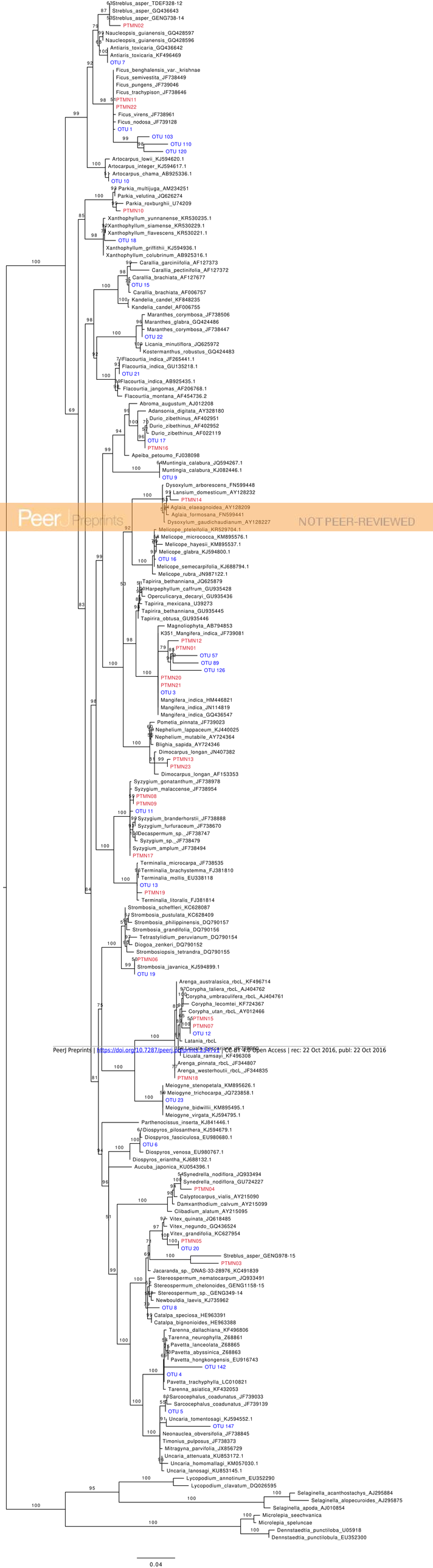
## Figure 4(on next page)

Maximum likelihood phylogenetic tree depicting the evolutionary relationship among identified OTUs from flying fox droppings, *rbcL* sequences obtained from individually collected leaf samples, and from public databases.

Values in nodes indicate ultrafast bootstrap support values and nodes with less than 50% support were collapsed. Scale bar indicates number of substitution per site.



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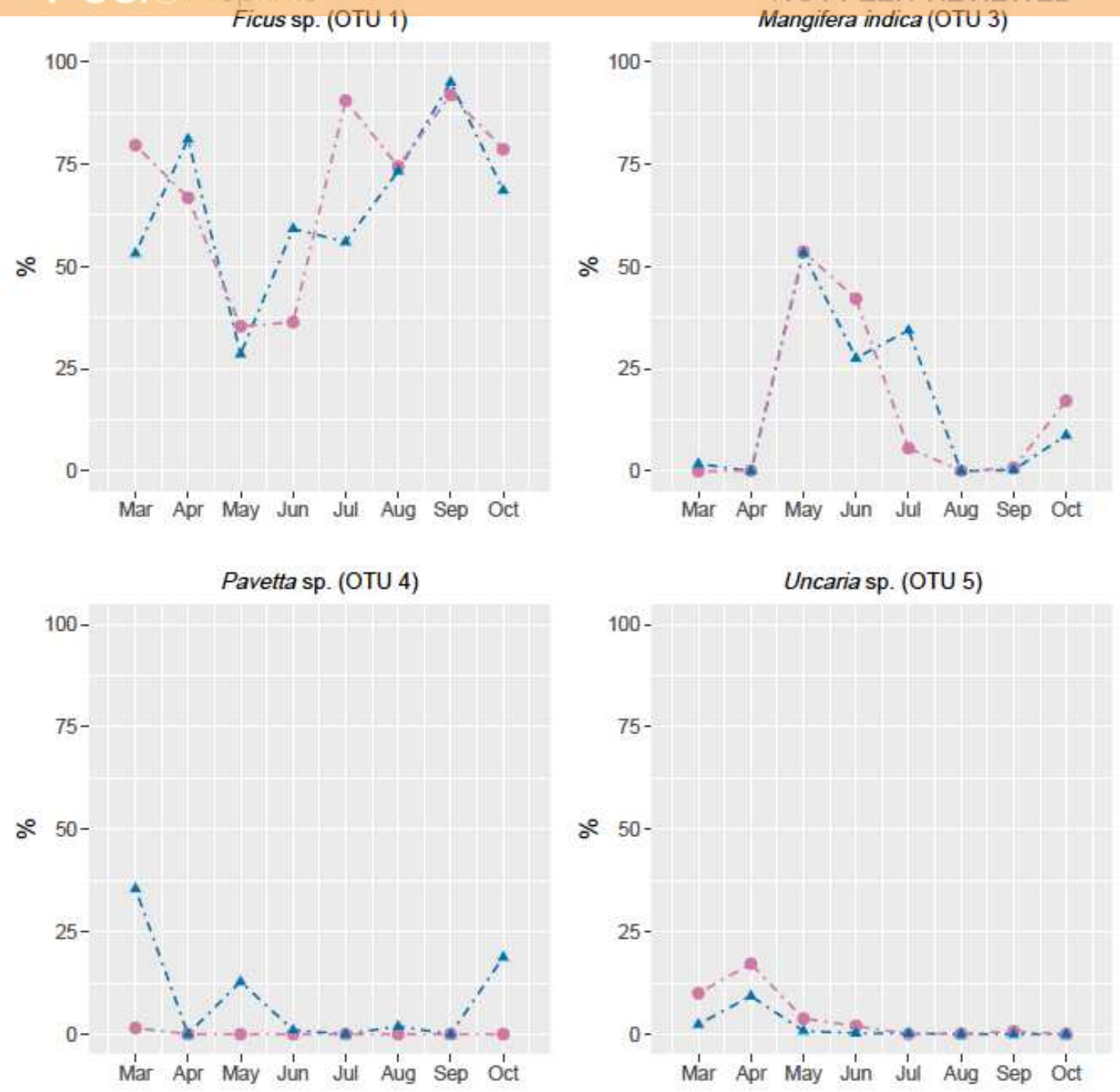
## Figure 5(on next page)

Spatio-temporal trends in consumption of the top four most dominant plant taxa detected in *Pteropus hypomelanus* droppings during March-October 2015 through NGS analysis.

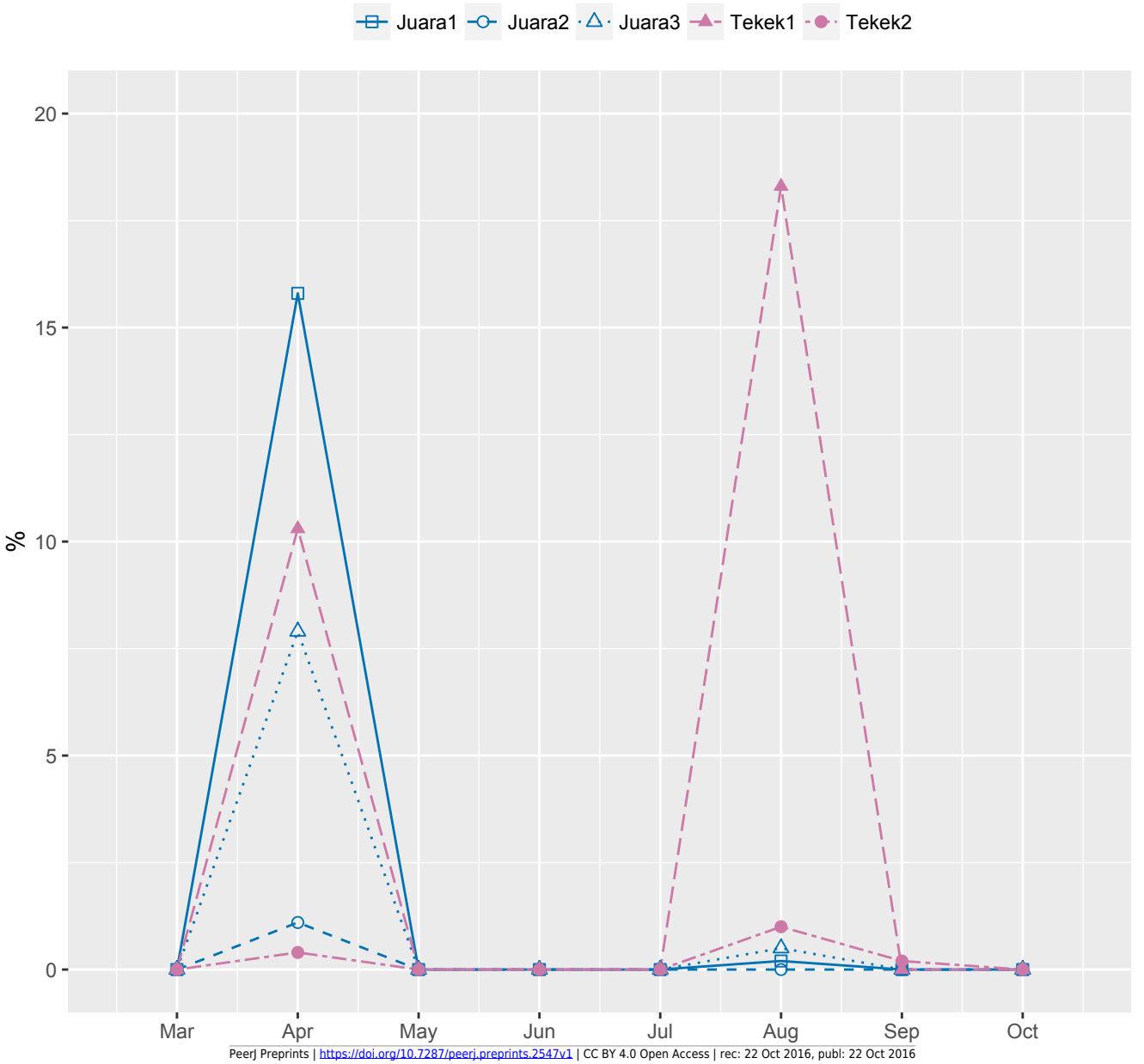


## Figure 6(on next page)

Spatio-temporal trends in consumption of *Antiaris* sp. (OTU 7) showing differences between roosts during March-October 2016, suggesting possible inter-roost variation in diet.

*Antiaris* sp. (OTU 7)



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## Table 1(on next page)

Summary information of 19 botanical specimens obtained from Tioman Island, Peninsular Malaysia.

| No. | Botanical Specimen ID | GPS coordinates | Specimen code | GenBank Accession code | Closest taxon match from BOLD database |
|---|---|---|---|---|---|
| 1 | *Anacardium occidentale* | N2° 47.756' E104° 12.220' | PTMN12 | KX618219 | Anacardiaceae |
| 2 | *Arenga pinnata* | N2° 48.048' E104° 11.823' | PTMN18 | KX618224 | *Arenga* sp. |
| 3 | *Cocus nucifera* | N2° 47.652' E104° 12.176' | PTMN07 | KX618214 | Arecaceae |
| 4 | *Durio zibethinus* | N2° 47.462' E104° 12.047' | PTMN16 | KX618222 | *Durio* sp. |
| 5 | *Euphoria malaiense* | N2° 47.300' E104° 12.139' | PTMN13 | KX618220 | Sapindaceae |
| 6 | *Ficus* sp. 1 | N2° 48.197' E104° 11.566' | PTMN11 | KX618218 | *Ficus* sp. |
| 7 | *Ficus* sp. 2 | N2° 49.354' E104° 10.145' | PTMN22 | KX618228 | *Ficus* sp. |
| 8 | *Lansium parasiticum* | N2° 48.012' E104° 11.906' | PTMN14 | KX618221 | *Lansium* sp. |
| 9 | *Mangifera indica* | N2° 47.645' E104° 12.176' | PTMN20 | KX618226 | *Mangifera* sp. |
| 10 | *Mangifera odorata* | N2° 48.134' E104° 11.745' | PTMN01 | KX148479 | Anacardiaceae |
| 11 | *Nephelium lappaceum* | N2° 49.353' E104° 09.916' | PTMN23 | KX618229 | Sapindaceae |
| 12 | *Parkia speciosa* | N2° 48.595' E104° 10.758' | PTMN10 | KX618217 | *Parkia* sp. |
| 13 | *Streblus asper* | N2° 26.214' E103° 50.857' | PTMN02 | KX618211 | *Streblus* sp. |
| 14 | *Strombosia* sp. | N2° 48.737' E104° 10.537' | PTMN06 | KX618213 | *Strombosia* sp. |

| | | | | | |
|---|---|---|---|---|---|
| 15 | *Syzygium malaccense* | N2° 47.406' E104° 12.096' | PTMN08 | KX618215 | *Syzygium* sp. |
| 16 | *Syzygium* sp. 1 | N2° 47.740' E104° 12.218' | PTMN09 | KX618216 | *Syzygium* sp. |
| 17 | *Syzygium* sp. 2 | N2° 47.965' E104° 11.983' | PTMN17 | KX618223 | *Syzygium* sp. |
| 18 | *Terminalia catappa* | N2° 47.615' E104° 12.190' | PTMN19 | KX618225 | *Terminalia* sp. |
| 19 | *Vitex pinnata* | N2° 47.745' E104° 12.225' | PTMN05 | KX618212 | *Vitex* sp. |

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## Table 2(on next page)

Primers used in this study for the amplification of rbcL from flying fox droppings. Bold, target sequence; underlined, Illumina partial adapter

| Primer Name | Sequence |
|---|---|
| rbcLaf-M13 | TGTAAAACGACGGCCAGT**ATGTCACCACAAACAGAGACTAAAGC** |
| rbcLa-revM13 | CAGGAAACAGCTATGAC**GTAAAATCAAGTCCACCRCG** |
| rbcL-357F | **CATTGTRGGTAATGTATTTGG** |
| rbcL-556R | **ACATTCATAAACHGCYCTACC** |
| IlluM-rbcLF | TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG**CATTGTRGGTAATGTATTTGG** |
| IlluM-rbcLR | GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG**ACATTCATAAACHGCYCTACC** |

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## Table 3(on next page)

Identities of 29 OTUs detected in flying fox droppings based on matches with reference database and botanical specimen DNA sequences across 8 months (Mar-Oct 2015) in Tioman Island, Malaysia. Number of sequencing reads, percentage identity hits and specim

| OTU no. (no. of reads) | Plant Family | Closest taxon match from BOLD (% identity hit) | Closest taxon match from NCBI (% identity hit) | Probable genus* | Closest botanical specimen match (code) |
|---|---|---|---|---|---|
| OTU 1 (2652) | Moraceae | *Ficus variegata* (100%) | *Ficus elastica* (100%) | *Ficus* | *Ficus* sp.2 (PTMN22) |
| OTU 3 (617) | Anacardiaceae | *Mangifera indica* (100%) | *Mangifera indica* (100%) | *Mangifera* | *Mangifera indica* (PTMN20) |
| OTU 4 (213) | Rubiaceae | *Coptosperma sp.* (100%) | *Pavetta indica* (100%) | *Pavetta* | |
| OTU 5 (106) | Rubiaceae | *Uncaria macrophylla* (100%) | *Uncaria attenuata* (100%) | *Uncaria* | |
| OTU 6 (67) | Ebenaceae | *Diospyros fasciculosa* (100%) | *Diospyros pilosanthera* (100%) | *Diospyros* | |
| OTU 7 (56) | Moraceae | *Antiaris toxicaria* (100%) | *Antiaris toxicaria* (100%) | *Antiaris* | |
| OTU 8 (80) | Bignoniaceae | *Anemopaegma album* (98.88%) | *Stereospermum annamense* (100%) | *Stereospermum* | |
| OTU 9 (24) | Muntingiaceae | *Muntingia calabura* (100%) | *Muntingia calabura* (100%) | *Muntingia* | |
| OTU 10 (9) | Moraceae | *Artocarpus heterophyllus* (100%) | *Artocarpus lakoocha* (100%) | *Artocarpus* | |
| OTU 11 (17) | Myrtaceae | *Syzygium malaccense* (100%) | *Backhousia sp.* (100%) | *Syzygium* | |
| OTU 12 (17) | Arecaceae | *Howea belmoreana* (100%) | *Roystonea oleracea* (100%) | - | *Cocos nucifera* (PTMN07) |
| OTU 13 (16) | Combretaceae | *Terminalia microcarpa* (100%) | *Terminalia catappa* (100%) | *Terminalia* | |
| OTU 15 (15) | Rhizophoraceae | *Carallia brachiata* (100%) | *Carallia brachiata* (100%) | *Carallia* | |
| OTU 16 (5) | Rutaceae | *Melicope elleryana* (100%) | *Pitaviaster haplophyllus* (100%) | - | |
| OTU 17 (9) | Malvaceae | *Durio zibethinus* (100%) | *Durio zibethinus* (100%) | *Durio* | *Durio zibethinus* (PTMN16) |
| OTU 18 (5) | Polygalaceae | *Xanthophyllum papuanum* (98.88%) | *Xanthophyllum yunnanense* (99%) | *Xanthophyllum* | |
| OTU 19 (3) | Olacaceae | *Maburea trinervis* (99.44%) | *Strombosia javanica* (100%) | *Strombosia* | *Strombosia* sp. (PTMN06) |
| OTU 20 (2) | Lamiaceae | *Vitex cofassus* (100%) | *Vitex peduncularis* (100%) | *Vitex* | *Vitex pinnata* (PTMN05) |

| | | | | |
|---|---|---|---|---|
| OTU 21 (5) | Salicaceae | *Flacourtia indica* (100%) | *Flacourtia indica* (100%) | *Flacourtia* |
| OTU 22 (4) | Chrysobalanaceae | *Maranthes glabra* (100%) | *Maranthes kerstingii* (100%) | *Maranthes* |
| OTU 23 (3) | Annonaceae | *Pseuduvaria froggattii* (100%) | *Pseuduvaria indochinensis* (100%) | *Pseuduvaria* |
| OTU 57 (26) | Anacardiaceae | *Faguetia falcata* (97.19%) | *Mangifera odorata* (98%) | *Mangifera* |
| OTU 89 (3) | Anacardiaceae | *Mangifera indica* (97.04%) | *Mangifera odorata* (98%) | *Mangifera* |
| OTU 103 (10) | Moraceae | *Ficus variegata* (96.63%) | *Ficus elastica* (97%) | *Ficus* |
| OTU 110 (23) | Moraceae | *Drypetes roxburghii* (95.51%) | *Drypetes roxburghii* (96%) | - |
| OTU 120 (7) | Moraceae | *Ficus variegata* (96.43%) | *Ficus religiosa* (96%) | *Ficus* |
| OTU 126 (2) | Anacardiaceae | *Schinus molle* (97.19%) | *Mangifera indica* (97%) | *Mangifera* |
| OTU 142 (4) | Rubiaceae | *Coptosperma nigrescens* (97.62%) | *Coptosperma rhodesiacum* (98%) | - |
| OTU 147 (2) | Rubiaceae | *Uncaria tomentosa* (96.61%) | *Uncaria tomentosa* (97%) | *Uncaria* |

NB: OTU numbers are not in running sequence because OTUs that were possible chimeras were removed from filtering process

*Based on known geographical occurrences in Malaysia with reference to plant distribution information from Flora Malesiana (http://floramalesiana.org/) and Wikipedia

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## Table 4(on next page)

Comparison of microscope vs. NGS analyses in detecting food plants present in 10 individual flying fox droppings collected on 6 May 2015.

*Not detected in the 8-month analysis, therefore no corresponding OTU number.

| Identified food plant | Microscope | | | | | | | | | | NGS | | | | | | | | | | OTU no. |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | |
| *Durio* sp. | x | | | | | | x | | x | | | | | | x | | | x | x | | 17 |
| *Ficus* sp. | | x | x | x | x | x | x | x | x | x | x | x | x | x | x | x | x | x | x | x | 1 |
| *Mangifera* sp. | | | | | | | | | | | x | x | x | x | x | x | x | x | x | x | 3 |
| *Strombosia* sp. | | | | | | | | | | | | | x | x | x | | | | x | | 19 |
| *Terminalia* sp. | | | | | | | | | | | x | x | | | x | x | x | | | | 13 |
| Arecaceae | | | | | | | | | | | x | x | x | x | x | x | x | x | x | x | 12 |
| *Uncaria* sp. | | | | | | | | | | | | x | | | | x | | | | | 5 |
| Sapindaceae | | | | | | | | | | | | x | | x | | x | x | | x | x | * |

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