Zoological Studies **61:**60 (2022)

Determining the Dietary Preferences of Wild Asian Elephants (*Elephas maximus*) in Taman Negara National Park Based on Sex and Age using trnL DNA Metabarcoding Analysis

Nurfatiha Akmal Fawwazah Abdullah-Fauzi[1], Kayal Vizi Karuppannan[2], Nor Hafisa Syafina Mohd-Radzi[1], Millawati Gani[1,2], Abd Rahman Mohd-Ridwan[1,3], Nursyuhada Othman[4,5], Hidayah Haris[4,5], Nur Hartini Sariyati[4,5], Nor Rahman Aifat[1,6], Muhammad Abu Bakar Abdul-Latiff[4,5], Mohd Firdaus Ariff Abdul-Razak[2], and Badrul Munir Md-Zain[1,*]

[1]*Department of Biological Sciences and Biotechnology, Faculty of Science and Technology, Universiti Kebangsaan Malaysia, 43600 Bangi, Selangor, Malaysia. *Correspondence: E-mail: abgbadd1966@yahoo.com (Md-Zain). E-mail: fatihanafaf97@gmail.com (Abdullah-Fauzi); E-mail: norhafisasyafina@gmail.com (Mohd-Radzi)*

[2]*Department of Wildlife and National Parks (PERHILITAN), KM 10 Jalan Cheras, 56100 Kuala Lumpur, Malaysia. E-mail: kayalvizi@wildlife.gov.my (Karuppannan); millawati@wildlife.gov.my (Gani); mfirdaus@wildlife.gov.my (Abdul-Razak)*

[3]*Centre for Pre-University Studies, Universiti Malaysia Sarawak, 94300 Kota Samarahan, Sarawak, Malaysia. E-mail: armridwan@unimas.my (Mohd-Ridwan)*

[4]*Faculty of Applied Sciences and Technology, Universiti Tun Hussein Onn Malaysia, 84600 Johor, Malaysia. E-mail: syuothman@gmail.com (Othman); hidyharis@gmail.com (Haris); hartini3297@gmail.com (Sariyati); latiff@uthm.edu.my (Abdul-Latiff)*

[5]*Oasis Integrated Group (OIG), Institute for Integrated Engineering (I^2E), Universiti Tun Hussein Onn Malaysia, 86400 Parit Raja, Johor, Malaysia*

[6]*Faculty of Tropical Forestry, Universiti Malaysia Sabah (UMS), Jalan UMS, 88400 Kota Kinabalu, Sabah, Malaysia. E-mail: aifat89@gmail.com (Aifat)*

(Received 10 October 2021 / Accepted 11 August 2022 / Published -- 2022)
Communicated by Teng-Chiu Lin

The world's largest terrestrial mammal, Asian elephants are known to have enormous feeding needs. Several factors such as season, sex, age, and daily activities of elephants influence the amount of food required by an individual. Generally, captive elephants have a limited choice of food on a daily basis compared with that of elephants in the wild. Elephants in captivity are fed according to a prepared feeding schedule, whereas wild elephants are free to choose the type of plants that they consume in their natural habitat. In the past, ecological observations have been widely used to determine the diet of wild elephants. However, the molecular approach was never been carried out. In the present study, the aim was to; 1) identify the plant diet of wild Asian elephants in Taman Negara National Park (TNNP) according to their sex and age using high-throughput DNA metabarcoding 2) determine the dietary formulation of captive elephants based on the generated plant metabarcoding database. DNA was extracted from 24 individual fecal samples collected using noninvasive sampling techniques from TNNP and the National Elephant Conservation Centre

(NECC) Kuala Gandah. Seven pooled samples comprising of male adult, female adult, male subadult, female subadult, male juvenile, female juvenile, and captive elephant were amplified and sequenced targeting the trnL region (50–150 base pairs). The CLC Genomic Workbench and PAST 4.02 software were used for data analysis. In total, 24 orders, 41 families, 233 genera, and 306 species of plants were successfully detected in the diet of the Asian elephants. The most abundant plant genera consumed were *Sporobolus* (21.88%), *Musa* (21.48%), and *Ficus* (10.80%). Plant variation was lower in samples from male elephants than in those from female elephants. The plant species identified were correlated with the nutrient benefits required by elephants. Besides, adults and subadults consumed more plant species than were consumed by juvenile elephants. However, there are no significant difference between ages and the two sexes. The findings of this study can be used as guidance by the Department of Wildlife and National Parks for the management of captive elephants, especially in NECC Kuala Gandah.

Key words: Fecal, Captivity, Peninsular Malaysia, Next-generation sequencing.

Citation: Abdullah-Fauzi NAF, Karuppannan KV, Mohd-Radzi NHS, Gani M, Mohd-Ridwan AR, Othman N, Haris H, Sariyati NH, Aifat NR, Abdul-Latiff MAB, Abdul-Razak MFA, and Md-Zain BM. 2022. Determining the dietary preferences of wild asian elephants (*Elephas maximus*) in Taman Negara National Park based on sex and age using trnL DNA metabarcoding analysis. Zool Stud **61:**60.

BACKGROUND

Family Elephantidae consists of two remaining genera, which are the African elephant (*Loxodonta*) and the Asian elephant (*Elephas*). The population of Asian elephants are distributed across 13 countries spanning South and Southeast Asia (IUCN/SSC Asian Elephant Specialist Group 2017). This species has been categorized as endangered, as listed in the International Union for Conservation of Nature (IUCN) Red List (IUCN 2020). According to Saaban et al. (2011), the population of Asian elephants in Peninsular Malaysia was estimated at 1223–1677 individuals and can only be found in Pahang, Terengganu, Kelantan, Kedah, Perak, and Johor. Taman Negara National Park (TNNP) has an extensive area of primary rainforest and is home to the world's largest population of wild Asian elephants (Karuppannan et al. 2020; Saaban et al. 2020). With a total area of 4,343 km^2, TNNP has also become one of the main wild elephant translocation sites in Peninsular Malaysia (Saaban et al. 2011; Jambari et al. 2019). Using the molecular sexing method, Karuppannan et al. (2020) identified 86 males and 131 females in TNNP. From these individuals, 15, 78, and 124 were juveniles, subadults, and adults, respectively (Karuppannan et al. 2020).

According to Kumar et al. (2019), 14,000–16,000 Asian elephants are currently in captivity. In Peninsular Malaysia, captive elephants are distributed in two elephant conservation centers and seven zoos; the National Elephant Conservation Centre (NECC) Kuala Gandah in Pahang, Elephant Conservation Centre Sungai Deka in Terengganu, A'Famosa Safari Wonderland, Kenyir Elephant Conservation Village, Taman Rekreasi & Zoo Kemaman, Zoo Melaka, Zoo Negara, Zoo Taiping & Night Safari, and Zoo Negeri Johor. In each of these locations, the number of captive elephants ranges from 7–40 individuals.

Due to the alarmingly low population numbers of Asian elephants, in 2010 they were upgraded to a fully protected species in Peninsular Malaysia under the Wildlife Conservation Act 2010 (Act 716). The greatest threat to the population of wild Asian elephant comes from human activities and agricultural growth, which have led to human–elephant conflict (Choudhury et al. 2008; Karuppannan et al. 2019a). In addition, poaching and illegal trade of wild elephants is a major concern in many countries (Douglas-Hamilton 2009; Hedges 2012). Sukumar et al. (1998) stated that male elephants are more likely to be hunted for their valuable tusks. However, elephant are also hunted for their skin, which is used as an ingredient in Chinese medicine (Apinya 2018). For elephants in captivity, common concerns include nutritional intake and weight problems (Vanitha et al. 2008). Furthermore, economic issues can be a significant constraint when attempting to implement wildlife conservation plans (Bandara and Tisdell 2003). Given these issues, the Department of Wildlife and National Parks (PERHILITAN) together with stakeholders has established the National Elephant Conservation Action Plan (NECAP) for Asian elephants (DWNP 2013).

Elephas maximus are considered mega-herbivores with tremendous dietary requirements. Sukumar (2006) stated that elephants are able to consume 10% of their body weight and spend 12 to 18 hours a day just to eat. In previous research, conventional approaches such as field observations (Sukumar 1990; Godagama et al. 1999; Borah and Deka 2008; Joshi and Singh 2008), in-depth interviews (Godagama et al. 1999), and microhistological methods (Yamamoto-Ebina et al. 2016; Koirala et al. 2019) have been used to investigate the diet of the Asian elephant (Magintan et al. 2016). However, the practicality of these techniques has been questioned due to the limited results they produce (Suba et al. 2017). Asian elephants are mixed feeders that graze and forage for food in the wild (Chen et al. 2006). Compared with wild elephants, captive elephants assimilate fewer nutrients as their food choice is restricted. Generally, 68% of plants ingested by wild Asian elephants were reported made up from seven types of families namely Fabaceae, Poaceae, Malvaceae, Sterculiaceae, Tiliaceae, Palmae and Cyperaceae (Sukumar 1990; Harich et al. 2016; Koirala 2017). In tropical rainforests, most Asian elephant diets are dominated by woody and fruiting plants such as grasses, shrubs, herbs and even roots (Sukumar 2006; Ullrey et al. 1997).

Meanwhile in captivity, elephants are given food in the form of pellets, fruits, vegetables as well as supplements such as vitamins and minerals (Benz 2005). However, to date, no studies have been conducted on the plant diet of Asian elephants according to their sex and age specifically.

Generally, previous studies involving herbivorous diets by sex and age have been poorly studied and very minimal. Due to different physiological states such as pregnancy and lactation processes in female animals, elephants are found to require higher levels of nutrients than males (Stokke 1999). Apart from that, size of an animal also plays a part in the selection of dietary preferences. This can be proven in a study on ruminant and non-ruminant herbivores, which indicates that fiber intake of an animal correlated with their body size (Demment and Van Soest 1985). Differences in dietary preferences among age groups can be seen in red kangaroos, *Osphranter rufus*, where the results stated that juveniles require more sustenance in their diet compared to other age classes. The main diet of this herbivore is also said to be dominated by grasses (Dawson et al. 2021).

The DNA metabarcoding method is a powerful tool for identifying a wide variety of plants that have been consumed by animals (Soininen 2012); indeed, it is known to be more accurate than the observation method. It is often used to analyze animal diet from fecal samples because this method avoids the handling of animals and can provide reliable results (Elliza et al. 2015; Aifat et al. 2016a; Hawlitschek et al. 2018; Md-Zain et al. 2018; Karuppannan et al. 2019b c). The trnL (UAA)-P6 marker from chloroplast DNA is a widely used as a marker in dietary analysis (Taberlet et al. 2007; Pompanon et al. 2012; Reese et al. 2019); it has a short length, conserved primer sites, and interspecific variation (Taberlet et al. 2007; Pompanon et al. 2012; Reese et al. 2019). Despite the reliability of the DNA metabarcoding approach, it has yet to be tested on Asian elephants.

In Peninsular Malaysia, the dietary intake of elephants has yet to be investigated using DNA metabarcoding analysis. In addition, knowledge of Asian elephant diet according to their sex and age is poorly understood. Furthermore, captive elephants in National Elephant Conservation Centre (NECC) Kuala Gandah require management of balanced diet that is appropriate to their sex and age, similar to in their natural habitat. Thus, the present study aimed to identify the preferences plant diet of wild Asian elephants in TNNP according to their sex and age using high-throughput DNA metabarcoding. In addition, it was predicted the diets consumed by wild elephants through DNA metabarcoding analyses differ in terms of plant diversity. Later, the generated plant database would help to determine the optimal diet formulation for elephant in captivity. Such a diet would promote the growth of captive elephants because they would receive the complete range of nutrients required from an early age. Overall, the findings of this study help distinguish the diet of wild Asian elephants based on sex and age. Moreover, the study is conducted in line with the NECAP strategy according to its long-term goals, i.e., goal number seven. This goal is to ensure that the captive

elephant population of Malaysia is well-managed and contributes to wild elephant conservation (DWNP 2013).

MATERIALS AND METHODS

Sample Collection

In total, 24 fecal samples collected in a noninvasive manner were used in this study; 18 samples from TNNP and six samples from the NECC Kuala Gandah (Table 1). Samples from the wild (TNNP) were obtained from the Wildlife Genetics Research Bank, PERHILITAN which were utilized from previous collected fecal samples. In contrast, the samples from captivity (NECC) were fresh and act as a positive control in this study, where the diet of captive elephants were already known. The collected fecal samples were preserved in absolute ethanol and maintained at room temperature.

Table 1. List of samples used in this study

No	Sample ID	Pooled samples	Locality
1	EM616	Male adult (MA)	Taman Negara National Park
2	EM705	Male adult (MA)	Taman Negara National Park
3	EM594	Male adult (MA)	Taman Negara National Park
4	EM707	Female adult (FA)	Taman Negara National Park
5	EM618	Female adult (FA)	Taman Negara National Park
6	EM640	Female adult (FA)	Taman Negara National Park
7	EM628	Male subadult (MSA)	Taman Negara National Park
8	EM729	Male subadult (MSA)	Taman Negara National Park
9	EM561	Male subadult (MSA)	Taman Negara National Park
10	EM566	Female subadult (FSA)	Taman Negara National Park
11	EM696	Female subadult (FSA)	Taman Negara National Park
12	EM677	Female subadult (FSA)	Taman Negara National Park
13	EM613	Male juvenile (MJ)	Taman Negara National Park
14	EM739	Male juvenile (MJ)	Taman Negara National Park
15	EM603	Male juvenile (MJ)	Taman Negara National Park
16	EM758	Female juvenile (FJ)	Taman Negara National Park
17	EM573	Female juvenile (FJ)	Taman Negara National Park
18	EM735	Female juvenile (FJ)	Taman Negara National Park
19	EM1540	Captivity (C)	NECC Kuala Gandah
20	EM1534	Captivity (C)	NECC Kuala Gandah
21	EM1524	Captivity (C)	NECC Kuala Gandah
22	EM1525	Captivity (C)	NECC Kuala Gandah
23	EM1530	Captivity (C)	NECC Kuala Gandah
24	EM1535	Captivity (C)	NECC Kuala Gandah

Sex and Age Determination of Wild Elephants

In this study, male and female elephants were distinguished using a non-invasive molecular sexing technique, targeting two Y-specific and one X-specific primers. This approach has been done previously by Karuppannan et al. (2019) using fecal samples from TNNP. Meanwhile, the age of wild elephants in TNNP was determined using their mean boli circumference (Reilly 2002; Tyson et al. 2002; Karuppannan et al. 2020). The measurements are listed in table 2.

Table 2. Measurements of mean boli circumference (mbc)

Measurements	Category
mbc ≤ 30 cm	juvenile
30 cm < mbc ≤ 42 cm	subadult
mbc > 42 cm	adult

DNA Extraction, Quantification, and Amplification

DNA was extracted from ~200-mg dung samples using a QIAGEN QIAamp Fast DNA Stool Mini Kit. Extracted DNA was then quantified using a NanoDrop spectrophotometer. All 24 samples were pooled to form 7 samples as follows; male adult, female adult, male subadult, female subadult, male juvenile, female juvenile, and captive elephant samples.

Polymerase chain reaction (PCR) was performed targeting the trnL region of the chloroplast gene (Taberlet et al. 2007). The PCR mixture contained 10 μL of Promega GoTaq Green Master Mix, 1 μL of each forward and reverse primer (10 μM), 6 μL of nuclease-free water, and 2 μL of DNA (final volume: 20 μL). The extracted samples were amplified using a Bio-Rad T100 Thermal Cycler under the following conditions: 95°C for 5 min, followed by 35 cycles of 95°C for 1 min, 56°C for 30 s, and 72°C for 1 min, with a final extension step at 72°C for 5 min. The PCR products were visualized using gel electrophoresis with 1% agarose gel in 1x TAE buffer to measure amplicon size. The laboratory work conducted in this study was performed at the National Wildlife Forensic Laboratory, PERHILITAN, Peninsular Malaysia.

Library Preparation and Sequencing

All PCR products of seven pooled samples with a volume of 10 μL each were sent to GeneSeq Sdn. Bhd for library preparation and sequencing. They were purified using SPRI Beads (Oberacker et al. 2019), which was followed by index PCR to incorporate Illumina dual index barcodes. The barcoded libraries were then pooled and gel-purified using a WizPrep Gel/PCR

Purification Mini Kit (WizBio, Korea) according to the manufacturer's instructions. The pooled libraries were quantified using a Denovix dsDNA High Sensitivity Assay and an appropriate amount of the libraries was loaded onto an iSeq100 (Illumina, San Diego) for 2 × 150 paired-end sequencing.

Data Analysis

Quality filtering and demultiplexing of sequences was conducted using CLC Genomic Workbench software (CLC) (Qiagen, USA). A preliminary assessment of quality scores across the Illumina data was performed using FASTQ files. The operational taxonomic units (OTUs) were clustered at 97% similarity and represented by a single sequence, which was aligned using the MUSCLE tool in CLC. The phylogenetic classification of the OTUs was conducted against a trnL database downloaded from GenBank. The alpha diversity indices (Shannon and Chao-1 index estimators) assessed the species richness in the elephants' diet and were generated using PAST 4.02 software (Hammer et al. 2001). The beta diversity was used to describe the dissimilarities in dietary diversity among individual elephants and was measured based on a UniFrac distance of 0.5.

A Venn diagram was produced to determine the shared and unique OTUs among the elephant samples at 97% similarity. To assess dietary diversity relationships among elephant samples, a heatmap was constructed using 1000 bootstrap replications following the Bray-Curtis distance. The correlation in diet (plant genera) among individual elephants from different populations was assessed using Pearson correlation coefficients. Correlations were visualized using cold-hot plots generated in PAST 4.02. Statistical significance was set at $P < 0.05$. Data analysis was conducted at the Evolutionary and Conservation Genetic Laboratory of Department of Technology and Natural Resources, Universiti Tun Hussein Onn Malaysia.

RESULTS

NGS Data Analysis

The concentration of extracted DNA was 1.4–10.5 ng/μL and the amplification products were 100–150-bp long (gained from trnL fragments). High-throughput DNA metabarcoding was used to successfully determine the diet of wild Asian elephants in TNNP according to sex and age; 371,556 plant sequences were obtained, ranging from 135,035 to 236,521 and from 63,361 to 202,372, respectively. After clustering at the 97% cut-off value, female elephants had more OTUs

(757), when compared with those of male elephants (Table 3). Among the age categories, subadult has the most OTUs (594), followed by adult (412), and juvenile (336) (Table 4).

Table 3. Number of sequences, OTUs, and unique OTUs of plants consumed by male and female Asian elephants

Samples	Sequences	OTUs	Unique OTUs
Male	135,035	504	251
Female	236,521	757	504
Total	371,556	1,261	

Table 4. Number of sequences, OTUs, and unique OTUs of plants consumed by adult, subadult, and juvenile Asian elephants

Samples	Sequences	OTUs	Unique OTUs
Adult	105,823	412	222
Subadult	202,372	594	384
Juvenile	63,361	336	142
Total	371,556	1,342	

Plant Species Identification

The plants consumed by *Elephas maximus* were successfully classified by taxonomic level into 24 orders, 41 families, 233 genera, and 306 species (Table 5).

Table 5. Total number of plants identified at different taxonomic levels according to trnL gene analysis

Taxonomic level	Total number
Order	24
Family	41
Genus	233
Species	306

Figures 1–3 show the relative abundance of dietary plants at the genus level according to analysis of wild Asian elephant samples from TNNP with categorization by sex and age. Overall, *Sporobolus* (21.88%), *Musa* (21.48%), *Ficus* (10.80%), *Laccosperma* (8.22%), *Coccothrinax* (6.15%), *Athrixia* (2.11%), *Colpothrinax* (1.73%), and *Mauritia* (1.26%) were the most dominant plant genera found in the diet of Asian elephants (Fig. 1). Considering sex and age, female and subadult Asian elephants consumed more plants than those in the other categories.

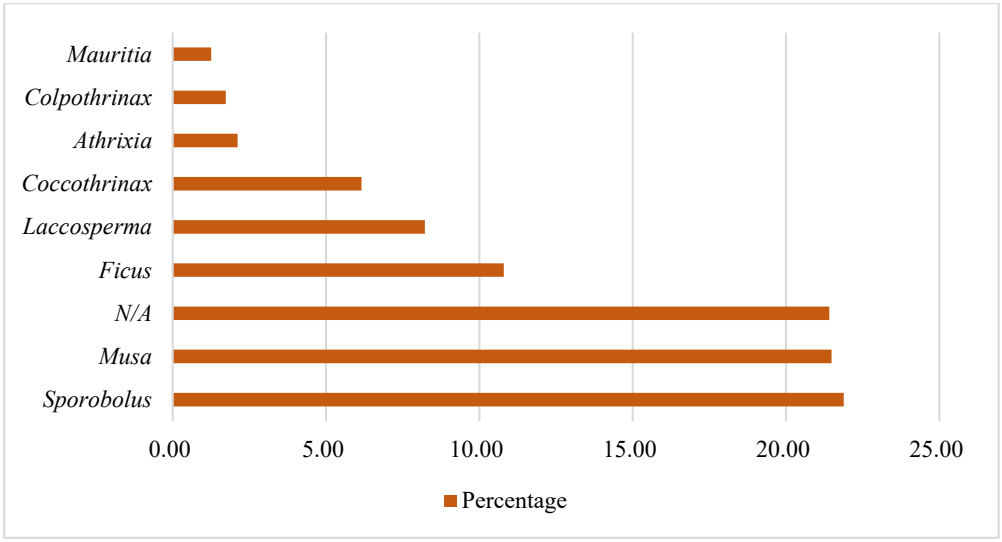

Fig. 1. Distribution (%) of plants consumed by wild Asian elephants in Taman Negara National Park (TNNP) at the genus level (> 1% abundance).

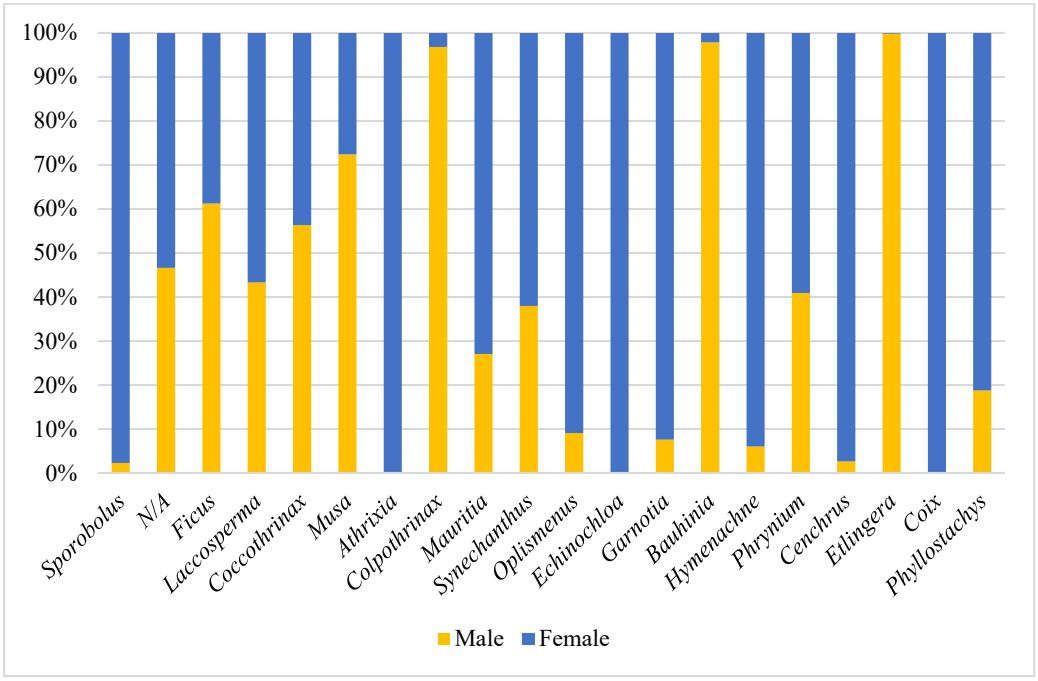

Fig. 2. Distribution (%) of plants consumed by male and female Asian elephants in Taman Negara National Park (TNNP) at the genus level (20 most abundant genera).

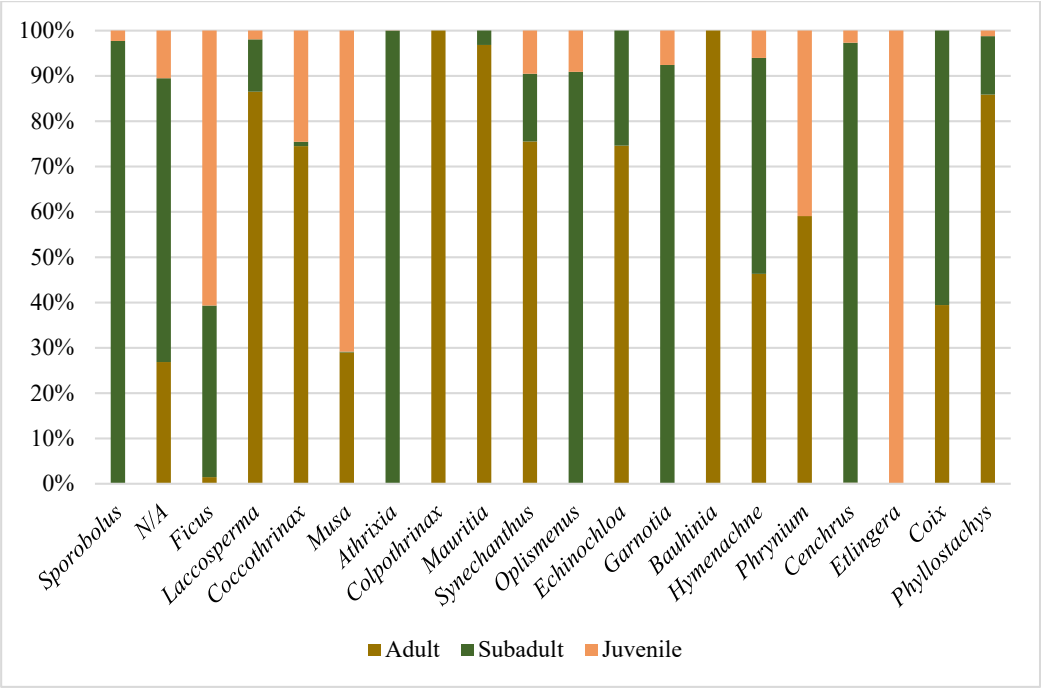

Fig. 3. Distribution (%) of plants consumed by adult, subadult, and juvenile Asian elephants in Taman Negara National Park (TNNP) at the genus level (20 most abundant genera).

Alpha Diversity Indices, Heatmap, Rarefaction Curve, and Venn Diagram

The alpha diversity (Shannon and Chao-1 indices) indicated that the diets of Asian elephants varied greatly depending on the analyzed parameters (Table 6 and 7). According to sex, male had a higher Shannon index value (H = 2.844) than that of female (H = 2.625). In contrast, female had a substantially greater Chao-1 value (785.5) than that of male (588.9). Among age categories, adult has the highest Shannon index value (H = 2.607), followed by subadult (H = 2.326), and juvenile (H = 2.225). However, the pattern of Chao-1 values was different: subadult had the highest value (624.7), followed by adult (459.4) and juvenile (396.6).

Table 6. Alpha diversity indices: Shannon and Chao-1 values for male and female Asian elephants

Samples	Shannon_H	Chao-1
Male	2.844	588.9
Female	2.625	785.5

Table 7. Alpha diversity indices: Shannon and Chao-1 values for adult, subadult, and juvenile Asian elephants

Samples	Shannon_H	Chao-1
Adult	2.607	459.4
Subadult	2.326	624.7
Juvenile	2.225	396.6

Figures 4 and 5 show the 20 most abundant plant genera present in male, female, adult, subadult, and juvenile Asian elephant samples. Darker colors indicate the more dominant genera.

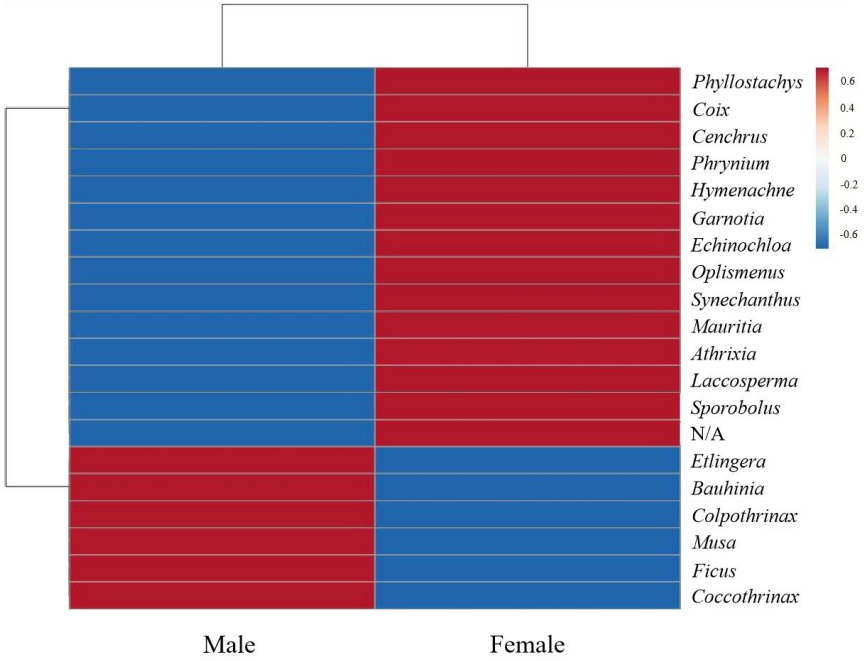

Fig. 4. Heatmap with a dendogram showing dietary plant abundance at the genus level for male and female Asian elephants. Gradient heatmap shows the 20 most abundant genera.

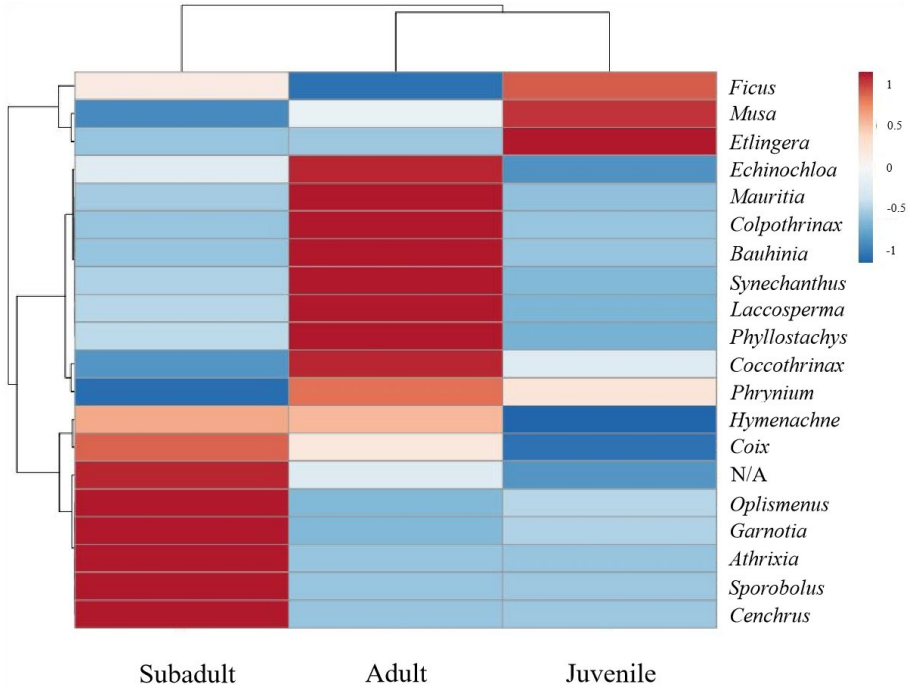

Fig. 5. Heatmap with a dendogram showing dietary plant abundance at the genus level for adult, subadult, and juvenile Asian elephants. Gradient heatmap shows the 20 most abundant genera.

The rarefaction curves in figures 6 and 7 show an increasing pattern for all samples used in this study, which indicates that plant richness was not adequately sequenced to facilitate identification of plant species. Indeed, the curves indicate that more accurate results would be possible if additional sampling were conducted.

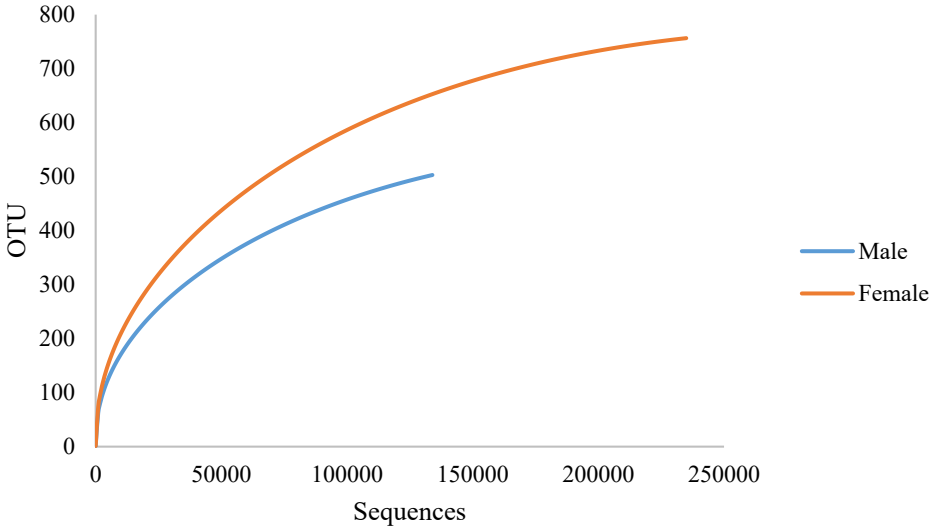

Fig. 6. Rarefaction curves for male and female Asian elephant samples.

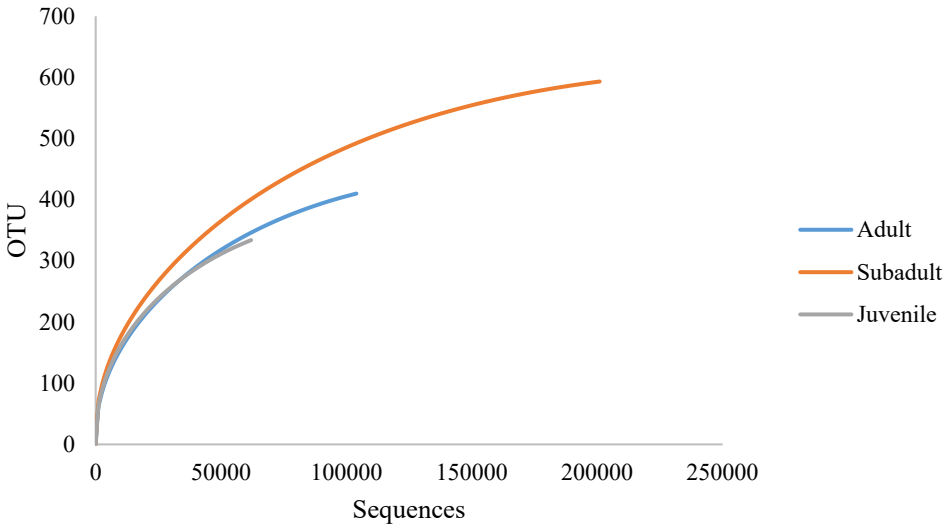

Fig. 7. Rarefaction curves for adult, subadult, and juvenile Asian elephant samples.

The Venn diagrams in Figures 8 and 9 show that 253 unique OTUs were shared between male and female Asian elephants (251 and 504 OTUs for male and female, respectively), whereas adult, subadult, and juvenile shared 74 OTUs.

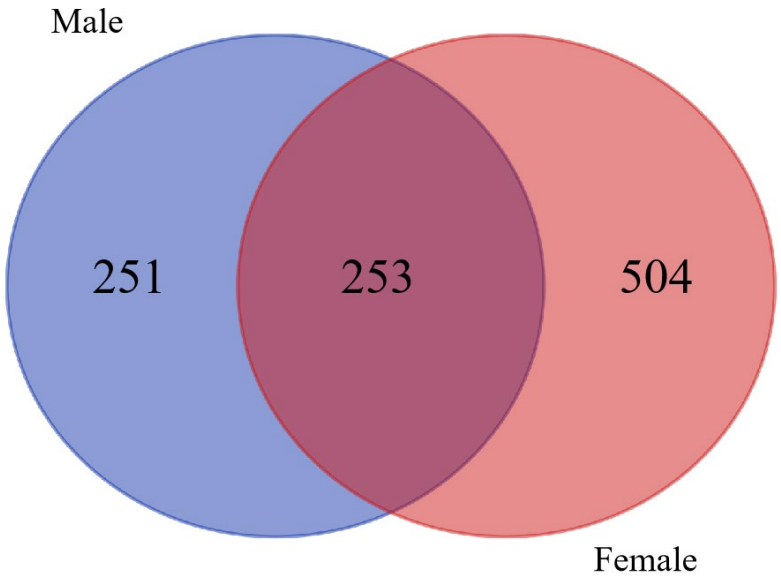

Fig. 8. Venn diagram showing the number of shared operational taxonomic units (OTUs) between male and female Asian elephants at 97% similarity.

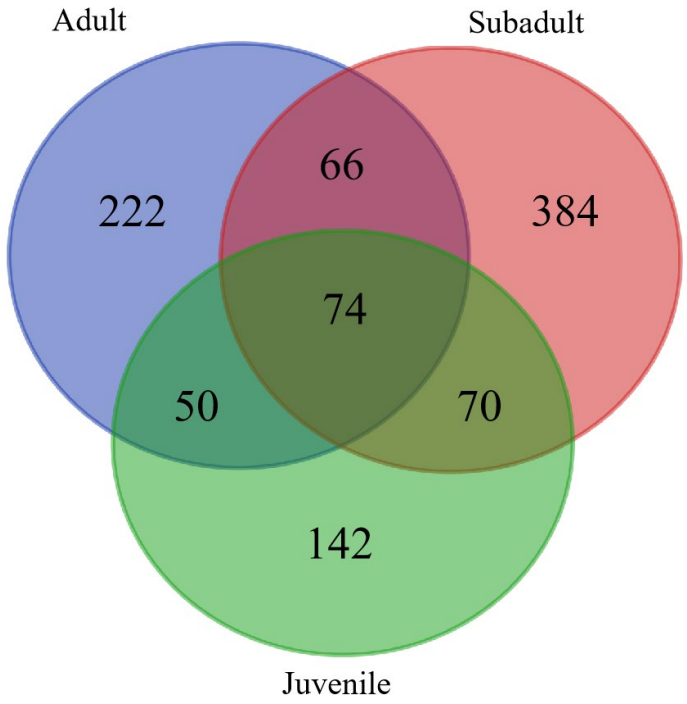

Fig. 9. Venn diagram showing the number of shared operational taxonomic units (OTUs) among adult, subadult, and juvenile Asian elephants at 97% similarity.

DISCUSSION

This study is the first to identify the diet of wild Asian elephants in TNNP according to sex and age, with gene analysis focused on the trnL region. The feces of wild elephants used in this study were collected from 2016 to 2017, which may have affected the sample quality and sequences generated from the samples. This has been demonstrated in previous studies where fresh fecal samples had high DNA quality and a higher percentage of sequencing reads (Hedges 2012; Syed-

Shabthar et al. 2013; Aifat et al. 2016b; Hawlitschek et al. 2018; Karuppannan et al. 2019b). Furthermore, the number of samples available for each parameter in this study was restricted. For individual samples with low DNA concentration, samples representing the same parameters and having low heterogeneity can be pooled, which also reduces costs and labor (Ray et al. 2019). Of the plant species identified in the analysis, some species, such as *Coccothrinax litoralis* and *Colpothrinax wrightii*, did not exist in Peninsular Malaysia; these were palm species that were endemic to Cuba (Leiva and Verdecia 2007; Henderson et al. 2019). This is because the trnL database used in the current study mainly includes plants from tropical rainforests in Africa and Madagascar. Thus, these detected plant species could be close relatives of the actual plant species ingested by Asian elephants in TNNP (Osman et al. 2020).

TNNP is a tropical evergreen rainforest with diverse flora and fauna. It is home to more than 3000 plant species and 150 mammal species (UNESCO 2021). Jambari et al. (2019) discovered that wild Asian elephants were dominant in the lowland forest compared with the highland forest of TNNP, which possesses the largest lowland tropical rainforests in Peninsular Malaysia. This finding relates to the number of plant species detected in the current study. By targeting the chloroplast trnL intron region, it was possible to identify 24 orders, 41 families, 233 genera, and 306 species of plants consumed by Asian elephants. The plant genera most abundantly consumed by wild Asian elephants in TNNP were *Sporobolus* (21.88%), *Musa* (21.48%), *Ficus* (10.80%), *Laccosperma* (8.22%), *Coccothrinax* (6.15%), *Athrixia* (2.11%), *Colpothrinax* (1.73%), and *Mauritia* (1.26%) (Fig. 1). While at family level, the diet of wild Asian elephants is dominated by the family Poaceae, Arecaceae, Moraceae, and Musaceae. *Sporobolus diandrus* and *Sporobolus indicus* were previously found in 10 states in Peninsular Malaysia including Pahang, Kelantan, and Terengganu, which is included in the TNNP area (Izzati et al. 2009). Furthermore, *Sporobolus* is a genus of grasses that is thought to be a primary food source for elephants (Dhairykar and Singh 2020).

Overall, the diet selection of wild Asian elephants in TNNP is general. This study found that female elephants have more plant homogeneity in their diet compared with that in the diet of male elephants. This is consistent with a study conducted on African elephants by Stokke (1999), who concluded that female elephants are more particular in foraging when compared with male elephants. These findings could be due to sexual dimorphism, *i.e.*, the body size hypothesis, which states that the larger an animal's size, the less selective it is in terms of foraging behavior (Stokke 1999; Woolley et al. 2011). In the present study, female Asian elephants in TNNP were found to have higher plant species richness in their diet relative to that in male diets. This may be related to the increased nutritional demands during pregnancy and lactation, *i.e.*, additional nutrients from a variety of plant species could be essential to these processes (Dierenfeld et al. 2020). Nevertheless, the diets of male Asian elephants were higher in plant diversity ($H = 2.844$) that those of females.

This may be due to their solitary existence, which involves foraging over a relatively larger area than that used by female elephants (Eisenberg 1980); this could contribute to the diversity of plants consumed by males. Generally, both sexes are likely to share similar dietary preferences but they differ in terms of the quantity of food consumed.

As reported by Koirala et al. (2019), the nutrient composition in the diet of elephants varies depending on their sex and age. However, the plant diet of wild elephants had not previously been analyzed according to age. In the current study, the number of OTUs detected in the samples showed the differences in diet among the age groups. Adult and subadult Asian elephants ingested more plant species than were ingested by adults. This is consistent with the requirement for growing elephants to consume relatively more food given the former's increased need for protein and other nutrients (Dhairykar and Singh 2020). Among the three age classes investigated here, adult elephants appeared to consume the most diverse range of plants, yet the plant richness is lower than in the subadults. We can conclude that, as Asian elephants grow older and begin to mature, they choose fewer types of plants and their diet becomes more specific. Nevertheless, most of the plant genera were shared among three age classes, although *Sporobolus* and *Oplismenus* were only present in subadults and juveniles. Even though elephants can be unspecialized feeders, they can also be quite particular in terms of selecting their preferred dietary plants (Swit 2016).

According to the captive elephants' caretaker, known as the mahout, captive elephants in NECC were fed with horse pellets in the morning. This is because their digestive system somewhat resembles that of a horse (Hatt and Clauss 2006). The elephants also are given a variety of plant species in the evening including *Musa* sp. (banana), *Cenchrus purpureus* (napier grass), *Citrullus lanatus* (watermelon), *Carica papaya* (papaya), and *Saccharum officinarum* (sugarcane). However, there was flexibility in the plant species provided that was entirely dependent on the availability of the stock. Similar to the plant species provided by the mahout, DNA metabarcoding analysis in this study identified an abundance of *Cenchrus purpureus*, *Musa* sp., and *Saccharum officinarum* in the diet of captive elephants in NECC. Plant species that are not given such as *Sporobolus diandrus* (tussock grass), *Ficus superba* (sea fig) and *Panicum auritum* (millet) also generated by the DNA metabarcoding, which may be eaten during walking hours around the NECC Kuala Gandah.

CONCLUSIONS

Final results of this study demonstrates that DNA metabarcoding able to detect the plants consumed by wild Asian elephants up to the species level. Despite being a preliminary study using stored fecal samples collected from the wild several years before, the association of diet with sex

and age was detected here. Nevertheless, extensive study will be needed to portray the dietary patterns of Asian elephants according to sex and age group. Further research such as nutrient analysis of plant species can be included to examine the plant composition eaten by elephant. A list of suitable dietary plant species has now been generated and this can be used by PERHILITAN as a reference to improve the plant diet of elephants in captivity. The knowledge gained from this study could also be applied to future practical research or conservation measures, particularly in the study of wild Asian elephants.

Acknowledgments: We gratefully acknowledge the Director General of the PERHILITAN Peninsular Malaysia, YBhg. Dato' Abdul Kadir bin Abu Hashim. We also expressed our gratitude to National Wildlife Forensic Laboratory (NWFL), Universiti Kebangsaan Malaysia (UKM) and Universiti Tun Hussein Onn Malaysia (UTHM) for their laboratory facilities throughout this project. We would like to thank our collaborators; Mr. Kaviarasu Munian from Forest Research Institute Malaysia (FRIM) and Dr. Wong Ee Phin from University of Nottingham Malaysia for their assistance throughout this project. This study was funded by Fundamental Research Grant Scheme (FRGS/1/2019/WAB13/NRE//1) provided by Ministry of Education Malaysia, and ST-2020-002 by Universiti Kebangsaan Malaysia.

Authors' contributions: NAF wrote the manuscript. NAF, KVK, NHS, MG, MFA conducted field sampling. NAF, KVK, HH, NHS, NO. NRA conducted laboratory work. NAF, ARMR, NHS. NRA conducted all the data analyses. KVK, ARMR, MAB, BMMZ critically revised the intellectual content. All authors read and approved the final version of the manuscript.

Competing interests: The authors agree that this research was conducted in the absence of any self-benefits, commercial or financial conflicts and declare absence of conflicting interests with the funders.

Availability of data and materials: Data in support of the findings of this study are available from the corresponding authors by reasonable request.

Consent for publication: All authors agree to its publication in Zoological Studies.

Ethics approval consent to participate: Department of Wildlife and National Parks Malaysia that provided with the necessary permission for this research (JPHL&TN(IP):100-34/1.24 Jld 16 (18).

REFERENCES

Aifat NR, Yaakop S, Md-Zain BM. 2016a. Optimization of partial Cyt *b* gene sequence from selected ancient *Presbytis* museum skin specimens. Malays Appl Biol **45:**93–96.

Aifat NR, Yaakop S, Md-Zain BM. 2016b. Ancient DNA analyses of museum specimens from selected *Presbytis* (Primate: Colobinae) based on partial Cyt *b* sequences. AIP Conf Proc **1784:**060024. doi:10.1063/1.4966862.

Apinya W. 2018. Jumbos surviving by the skin of their teeth. Available at: https://www.bangkokpost.com/thailand/general/1452643/jumbos-surviving-by-the-skin-of-their-teeth. Accessed 26 July 2021.

Bandara R, Tisdell C. 2003. Comparison of rural and urban attitudes to the conservation of Asian elephants in Sri Lanka: empirical evidence. Biol Conserv **110(3):**327–342. doi:10.1016/s0006-3207(02)00241-0.

Benz A. 2005. The elephant's hoof: macroscopic and microscopic morphology of defined locations under consideration of pathological changes. Doctoral Dissertation, University of Zurich, Switzerland.

Borah J, Deka K. 2008. Nutritional evaluation of forage preferred by wild elephants in the Rani Range Forest, Assam, India. Gajah **28:**41–43.

Chen J, Deng X, Zhang L, Bai Z. 2006. Diet composition and foraging ecology of Asian elephants in Shangyong, Xishuangbanna, China. Acta Ecol Sin **26(2):**309–316. doi:10.1016/s1872-2032(06)60006-1.

Choudhury A, Lahiri Choudhury DK, Desai A, Duckworth JW, Easa PS, Johnsingh AJT, Fernando P, Hedges S, Gunawardena M, Kurt F, Karanth U, Lister A, Menon V, Riddle H, Rübel A, Wikramanayake E. 2008. *Elephas maximus*: The IUCN Red List of Threatened Species 2008. IUCN SSC Asian Elephant Specialist Group.

Dawson TJ, Norton MA, Rodoreda S, Abbott SK, McLeod SR. 2021. The burden of size and growth for the juveniles of large mammalian herbivores: Structural and functional constraints in the feeding biology of juveniles relative to adults in red kangaroos, *Osphranter rufus*. Ecol Evol **11(13):**9062–9078. doi:10.1002/ece3.7750.

Demment MW, Van Soest PJ. 1985. A nutritional explanation for body-size patterns of ruminant and nonruminant herbivores. Am Nat **125(5):**641–672. doi:10.1086/284369.

Dhairykar M, Singh KP. 2020. Management of nutrition in captive Asian elephants. Int J Vet Sci Anim Husb **5(4):**160–163.

Dierenfeld ES, Han YA, Mar KU, Aung A, Soe AT, Lummaa V, Lahdenperä M. 2020. Milk composition of Asian elephants (*Elephas maximus*) in a natural environment in Myanmar during late lactation. Animals **10(4):**725. doi:10.3390/ani10040725.

Douglas-Hamilton I. 2009. The current elephant poaching trend. Pachyderm **45:**154–157.

DWNP. 2013. Department of Wildlife and National Parks Peninsular Malaysia, 2013. National Elephant Conservation Action Plan: Blueprint to Save Malaysian Elephants. Kuala Lumpur: Department of Wildlife and National Parks Peninsular Malaysia.

Eisenberg JF. 1980. Ecology and behavior of the Asian elephant. Elephant **1(5):**5. doi:10.22237/elephant/1521731835.

Elliza MN, Shukor MN, Othman N, Md-Zain BM. 2015. Haplotype distribution among endangered Asian elephants (*Elephas maximus*) in Peninsular Malaysia. Malays Appl Biol **44:**129–135.

Godagama WK, Wemmer C, Ratnasooriya WD. 1999. The Diet of Sri Lankan Domesticated Elephant (*Elephas maximus maximus*). Vidyodaya Journal of Science 1 **8:**75–85.

Hammer Ø, Harper DA, Ryan PD. 2001. PAST: paleontological statistics software package for education and data analysis. Palaeontol Electron **4(1):**9.

Harich FK, Treydte AC, Ogutu JO, Roberts JE, Savini C, Bauer JM, Savini T. 2016. Seed dispersal potential of Asian elephants. Acta Oecol **77:**144–151. doi:10.1016/j.actao.2016.10.005.

Hatt JM, Clauss M. 2006. Feeding Asian and African elephants *Elephas maximus* and *Loxodonta africana* in captivity. Int Zoo Yearb **40(1):**88–95. doi:10.1111/j.1748-1090.2006.00088.x.

Hawlitschek O, Fernandez-Gonzalez A, Balmori-de la Puente A, Castresana J. 2018. A pipeline for metabarcoding and diet analysis from fecal samples developed for a small semi-aquatic mammal. PLoS One **13(8):**1–19. doi:10.1371/journal.pone.0201763.

Hedges S. 2012. Estimating elephant population density and abundance from dung pile density: theoretical concepts. *In*: Hedges S (ed) Monitoring elephants and assessing threats: a manual for researchers, managers and conservationists. University Press (India) Private Limited, Himayatnagar, India.

Henderson A, Galeano G, Bernal R. 2019. Field guide to the palms of the Americas, vol 5390. Princeton University Press. https://doi.org/10.1515/9780691197708.

IUCN. 2020. The IUCN Red List of Threatened Species: *Elephas maximus*, Version 2020-2. Availale at: https://www.iucnredlist.org. Accessed June 2020.

IUCN/SSC Asian Elephant Specialist Group. 2017. Asian elephant range states meeting. Gajah: Regain Foundation, International Elephant Foundation, Forum Konservasi, Jakarta, Indonesia, April 18-20 2017.

Izzati MZNA, Sidique SNM, Ismail NA, Salleh B. 2009. Distribution and diversity of Fusarium species associated with grasses in ten states throughout Peninsular Malaysia. Biotropia **16(2):**55–64. doi:10.11598/btb.2009.16.2.57.

Jambari A, Sasidhran S, Halim HRA, Mohamed KA, Ashton-Butt A, Lechner AM, Azhar B. 2019. Quantifying species richness and composition of elusive rainforest mammals in Taman Negara National Park, Peninsular Malaysia. Glob Ecol Conserv **18:**e607. doi:10.1016/j.gecco.2019.e00607.

Joshi R, Singh R. 2008. Feeding behaviour of wild Asian elephants (*Elephas maximus*) in the Rajaji National Park. J Am Sci **4(2):**34–48.

Karuppannan KV, Aifat NR, Mohamed KA, Ahmad-Tahir NFD, Nordin FM, Yaakop S, Maldonado JE, Md-Zain BM. 2019a. Genetic variations among selected wild Asian elephant populations in Peninsular Malaysia based on mitochondrial D-loop region DNA sequences. Biodiversitas **20:**2494–2502. doi:10.13057/biodiv/d200910.

Karuppannan KV, Abdul-Razak MFA, Ahmad-Tahir NFD, Rosli N, Yaakop S. 2019b. Influence of Asian elephant dung decay on DNA recovery. J Sustain Sci Manag **14(5):**175–181.

Karuppannan KV, Mohamed KA, Keliang C, Naim NAM, Razak MFAA, Yaakop S, Md-Zain BM. 2019c. Sex determination among *Elephas maximus* faecal samples collected from selected sampling plots in Taman Negara National Parks, Peninsular Malaysia. AIP Conf Proc **2111(1):**060012. doi:10.1063/1.5111274.

Karuppannan KV, Mohamed KA, Abdul-Razak MFA, Ahmad-Tahir NFD, Mohd-Naim NA, Keliang C, Md-Zain BM. 2020. Sex ratio and age structure patterns of Asian elephants from Peninsular Malaysia revealed by non-invasive surveys. J Anim Plant Sci **30(6):**1415–1423. doi:10.36899/japs.2020.6.0163.

Koirala RK, Ji W, Paudel P, Coogan SC, Rothman JM, Raubenheimer D. 2019. The effects of age, sex and season on the macronutrient composition of the diet of the domestic Asian elephant. J Appl Anim Res. **47:**5–16.

Koirala RK. 2017. Nutritional ecology of Asian elephant (*Elephas maximus*) and human-wildlife interactions. PhD Dissertation, Massey University, Albany, New Zealand.

Kumar V, Pradheeps M, Kokkiligadda A, Niyogi R, Umapathy G. 2019. Non-invasive assessment of physiological stress in captive Asian elephants. Animals **9(8):**553. doi:10.3390/ani9080553.

Leiva A, Verdecia R. 2007. Conservation of Cuban palms. *In*: Building a sustainable future: the role of botanic gardens. Proceedings of the 3rd Global Botanic Gardens Congress, pp. 1–3.

Magintan D, Shukor MN, Lihan T, Campos AA, Saaban S, Husin SM, Ahmad MN. 2016. An assessment of elephant home ranges and movement patterns during construction of Hulu

Terengganu hydroelectric dam, Terengganu using GPS satellite collars. AIP Conf Proc **1784(1):**060006. doi:10.1063/1.4966844.

Md-Zain BM, Abid-Kamal SNA, Aifat NR, Abdul-Latiff MAB, Mohd-Hashim A, Ampeng A, Yaakop S, Samat A. 2018. Molecular identification of shark fins in Malaysian Borneo's local markets. Biodiversitas **19:**1035–1043.

Oberacker P, Stepper P, Bond DM, Höhn S, Focken J, Meyer V, Jurkowski TP. 2019. Bio-On-Magnetic-Beads (BOMB): Open platform for high-throughput nucleic acid extraction and manipulation. PLoS Biol **17(1):**e3000107. doi:10.1371/journal.pbio.3000107.

Osman NA, Abdul-Latiff MAB, Mohd-Ridwan AR, Yaakop S, Nor SM, Md-Zain BM. 2020. Diet composition of the wild stump-tailed macaque (*Macaca arctoides*) in Perlis State Park, Peninsular Malaysia, using a chloroplast tRNL DNA metabarcoding approach: a preliminary study. Animals **10(12):**2215. doi:10.3390/ani10122215.

Pompanon F, Deagle BE, Symondson WO, Brown DS, Jarman SN, Taberlet P. 2012. Who is eating what: diet assessment using next generation sequencing. Mol Ecol **21(8):**1931–1950. doi:10.1111/j.1365-294x.2011.05403.x.

Ray KJ, Cotter SY, Arzika AM, Kim J, Boubacar N, Zhou Z, Doan T. 2019. High-throughput sequencing of pooled samples to determine community-level microbiome diversity. Ann Epidemiol **39:**63–68. doi:10.1016/j.annepidem.2019.09.002.

Reese AT, Kartzinel TR, Petrone BL, Turnbaugh PJ, Pringle RM, David LA. 2019. Using DNA metabarcoding to evaluate the plant component of human diets: A proof of concept. MSystems **4(5)**. doi:10.1128/msystems.00458-19.

Reilly J. 2002. Growth in the Sumatran elephant (*Elephas maximus sumatranus*) and age estimation based on dung diameter. J Zool **258(2):**205–213. doi:10.1017/s0952836902001322.

Saaban S, Othman NB, Yasak MNB, Burhanuddin MN, Zafir A, Campos-Arceiz A. 2011. Current status of Asian elephants in Peninsular Malaysia. Gajah **35(1):**67–75.

Saaban S, Yasak MN, Gumal M, Oziar A, Cheong F, Shaari Z, Hedges S. 2020. Viability and management of the Asian elephant (*Elephas maximus*) population in the Endau Rompin landscape, Peninsular Malaysia. PeerJ **8:**8209. doi:10.7717/peerj.8209.

Syed-Shabthar SMF, Rosli MKA, Mohd-Zin NAA, Romaino SMN, Fazly-Ann ZA, Mahani MC, Abas-Mazni O, Zainuddin R, Yaakop S, Md-Zain BM. 2013. The molecular phylogenetic signature of Bali cattle revealed by maternal and paternal markers. Mol Biol Rep **40:**5165-5176.

Soininen EM. 2012. Interactions between small rodents and their food plants in tundra habitats. PhD Dissertation, UiT The Arctic University of Norway, Tromsø, Norway.

Stokke S. 1999. Sex differences in feeding-patch choice in a megaherbivore: elephants in Chobe National Park, Botswana. Can J Zool **77(11):**1723–1732. doi:10.1139/z99-145.

Suba RB, Beveridge NGP, Kustiawan W, De Snoo GR, De Iongh HH. 2017. Foraging ecology and diet of Bornean elephants (*Elephas maximus borneensis*) in the Sebuku forest area, North Kalimantan Province of Indonesia: Do the choices matter? Integr Zool **13:**219–223. doi:10.1111/1749-4877.12283.

Sukumar R, Ramakrishnan U, Santosh JA. 1998. Impact of poaching on an Asian elephant population in Periyar, southern India: a model of demography and tusk harvest. Anim **1(4):**281–291. doi:10.1111/j.1469-1795.1998.tb00039.x.

Sukumar R. 1990. Ecology of the Asian elephant in southern India. II. Feeding habits and crop raiding patterns. J Trop Ecol **6(1):**33–53. doi:10.1017/s0266467400004004.

Sukumar R. 2006. A brief review of the status, distribution and biology of wild Asian elephants *Elephas maximus*. Int Zoo Yearb **40(1):**1–8. doi:10.1111/j.1748-1090.2006.00001.x.

Swit N. 2016. The impacts of elephant grazing on plant succession in tropical forests of Africa. The Downtown Review **2(2):**8.

Taberlet P, Coissac E, Pompanon F, Gielly L, Miquel C, Valentini A, Willerslev E. 2007. Power and limitations of the chloroplast trnL (UAA) intron for plant DNA barcoding. Nucleic Acids Res **35(3):**e14. doi:10.1093/nar/gkl938.

Tyson M, Hedges S, Sitompul AF. 2002. WCS Indonesia Sumatran elephant project: Six-month report January–June, 2002. Wildlife Conservation Society, Jakarta, Indonesia.

Ullrey DE, Crissey SD, Hintz HF. 1997. Elephants: nutrition and dietary husbandry. *In*: Nutrition Advisory Group Handbook, pp. 1–20.

UNESCO National Park (Taman Negara) of Peninsular Malaysia. 2021. Taman Negara. Available at: https://whc.unesco.org/en/tentativelists/5927/2021. Accessed Sept. 2021.

Vanitha V, Thiyagesan K, Baskaran N. 2008. Food and feeding of captive Asian elephants (*Elephas maximus*) in the three management facilities at Tamil Nadu, south India. J Scl Trans Environ Technov **2(2):**87–97. doi:10.20894/stet.116.002.002.005.

Woolley LA, Page B, Slotow R. 2011. Foraging strategy within African elephant family units: why body size matters. Biotropica **43(4):**489–495. doi:10.1111/j.1744-7429.2010.00733.x.

Yamamoto-Ebina S, Saaban S, Campos-Arceiz A, Takatsuki S. 2016. Food habits of Asian elephants *Elephas maximus* in a rainforest of northern Peninsular Malaysia. Mammal Study **41(3):**155–161. doi:10.3106/041.041.0306.