- 2 Impact of Urbanisation and Agriculture on the Diet of Fruit Bats
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ABSTRACT

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The expansion of cities and agricultural plantations have unpredictable impacts on biodiversity and ecosystem services. Yet some species are capable of tolerating anthropogenic impacts and continue to provide ecological services in highly disturbed landscapes. The objective of this study was to use DNA barcoding to identify digested plant materials and seeds in the faeces of frugivorous bats (Cynopterus brachyotis) and investigate whether (1) C. brachyotis in urban and agricultural areas exploit cultivated and exotic plants as a novel food resource and as a consequence, potentially facilitate the invasion of cultivated and exotic plants, or whether (2) C. brachyotis exploit native plants and as a consequence, potentially promote forest regeneration. A native species, Ficus fistulosa, was the most frequently detected plant and the seeds were found in bat faeces from all sampling sites suggesting the potential of fruit bats in dispersing seeds. However, we also detected several exotic plants in the faeces of C. brachvotis which suggests that the fruit bats exploit novel food resources at all sites. We recorded a diverse diet of C. brachyotis at an oil palm plantation which indicated that the fruit bats are not predominantly feeding on oil palm fruits. By using DNA barcoding, we detected plants that have not been reported in previous studies of the diet of C. brachyotis, although we could not identify which part of the plant was being consumed by the fruit bats. Given the varied diet of C. brachyotis, the potential of this bat to adapt to changing landscapes is high and they are likely dispersing seeds of native pioneer plants (*Ficus*).

KEYWORDS

DNA barcoding, land use, oil palm plantation, seed dispersal, species' interaction, urbanisation

INTRODUCTION

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Between 2000 and 2010, the area of urban land expanded by more than 22% in East-Southeast 37 Asia (Schneider et al. 2015). In Peninsular Malaysia, urban land is expanding 1.5% annually 38 39 (Schneider et al. 2015), and the land area used for oil palm plantation is expanding 7% annually 40 (Butler 2013). Such changes in land use are often associated with alterations to biogeochemical 41 cycles, climate and biodiversity (Grim et al. 2008; Fitzherbert et al. 2008), for example, the introduction of exotic species in human-dominated areas (Grim et al. 2008; Fitzherbert et al. 2008) 42 43 which may compete with and extirpate native species (Faeth et al. 2005; McConkey et al. 2012). 44 However, despite losses of biodiversity, important ecological processes still take place in urban 45 and agricultural habitats. For example, botanical and residential gardens in urban areas provide 46 diverse food resources and nesting areas to bees (Sing et al. 2016) which pollinate garden plants, 47 while birds and bats continue to survive in urban areas and can provide critical seed dispersal services for native plants, particularly for pioneer species such as *Ficus* (Tan et al. 2000; Corlett 48 49 2005). Understanding how ecosystem services in human modified environments are maintained, albeit often involving exotic species and novel interactions (Corlett 2005), is a serious and 50 growing challenge. As a first step it is important to understand how a population uses resources 51 52 in natural versus human modified environments. 53 The Lesser Dog-faced Fruit Bat (a species complex often reported as *Cynopterus brachyotis*; Campbell et al. 2004; Wilson et al. 2014) is the most common bat in Peninsular Malaysia and is 54 abundant in primary and secondary forests, agricultural land, and urban areas (Campbell et al. 55 2004; Jayaraj et al. 2012). Because of its ubiquitous presence, C. brachyotis is an excellent model 56 of ecological flexibility with a potentially important role in seed dispersal. C. brachyotis has been 57

reported feeding on sixteen plant species in primary forest (Hodgkison et al. 2004), 66 plant 58 59 species in secondary forests (Tan et al. 1998) and 38 species in urban areas (Tan et al. 2000). 60 While C. brachyotis in urban areas demonstrated distinct food preferences during fruiting seasons (Tan et al. 2000), C. brachyotis in primary forest exploited both "steady state" and "big bang" 61 62 plants and has not shown variation in diet over time (Hodgkison et al. 2004). The apparent 63 flexibility of C. brachyotis in diet suggests a significant capacity to adapt to changing 64 environments. The flexible use of modified habitats may also bring fruit bats into conflict with 65 farmers in agricultural areas where bats may be perceived as foraging for food in cultivated commercial crops and consequently targeted as crop pests (Fujita and Tuttle 1991). 66 One limitation with previous research into fruit bat foraging preferences has been the inability to 67 identify fruit pulp and fragmented material in their faeces. Taxonomic assessment of fruit bats' 68 food resources has been restricted to observations during behavioural studies of bats which are 69 70 difficult in low light conditions (Phua and Corlett 1989), or morphology-based species 71 identification of seeds in faeces or plant remnants in masticated pellets (Tan et al. 1998; Hodgkison et al. 2004). One potential solution to this impediment is the use of molecular 72 73 methods such as DNA barcoding (Hebert et al. 2003; Wilson et al. 2016) which matches short DNA sequences of standardised regions (e.g. rbcL and ITS2 for plants; CBOL 2009; Chen et al. 74 75 2010) to taxonomically verified DNA sequences (Kuzmina et al. 2012). DNA barcoding has been 76 used to identify even the most degraded and digested material in the faeces of insectivorous (Clare et al. 2009) and frugivorous bats (Hayward 2013; Aziz et al. 2017). 77 78 The objective of this study was to use DNA barcoding to identify the digested plant materials and

seeds in the faeces of frugivorous bats (C. brachyotis) and investigate whether (1) C. brachyotis

in urban and agricultural areas adapt to the changing landscapes to exploit cultivated and exotic plants as a novel food resource and are thus are potential vectors of their dispersal or (2) whether *C. brachyotis* exploit native plants and as a consequence potentially promote forest regeneration.

METHODS

Ethics

- Faecal collection and bat sampling were conducted with authorization from Department of
 Wildlife and National Parks, Peninsular Malaysia (JPHLandTN(IP)100-34/1.24 Jld. 4(34)) and
 Institutional Animal Care and Use Committee, University of Malaya (ISB/10/06/2016/LVC (R)).

 Study sites and faecal sampling
 - We conducted faecal sampling at three sites with either urban, agricultural or secondary forest land use (Fig. 1). The urban site was an abandoned residential area located between University of Malaya and MAHSA University in Kuala Lumpur city in close proximity to a busy hospital and occupied residences. The agricultural site was located within a 2940 ha oil palm plantation (*Elaies guineensis x Elaies oleifera*) at Bemban, Melaka. The secondary forest site was located at the University of Malaya Field Studies Centre which is situated within 120 hectares of a secondary forest selectively logged from 1956 to 1958 (Medway 1966; Sing et al. 2013).
 - We collected fresh faeces from individual bats (*C. brachyotis* sensu stricto identified following Jayaraj et al. 2012) captured using mist nets at the urban site for eleven days between 10 June to 18 December 2015 and at the agricultural site for four days from 12 January to 15 January 2016.

Most of the bats defecated immediately when captured, but those that did not were kept in

individual cloth bags for one hour to produce faeces and were then released. The faeces collected 101 from one individual was considered as a single independent sample. 102 We located a roosting colony of C. brachyotis (identified by capturing and measuring four 103 individuals from the colony following Jayaraj et al. 2012) at the secondary forest site. The floor 104 105 below the roost was cleaned daily and fresh faeces from the colony were collected from the floor 106 non-invasively between 10 July and 25 September 2015. We treated each faecal sample (i.e. 107 collected into an individual Eppendorf tube) as an independent sample. The faeces were kept in 1.5 ml Eppendorf tubes filled with 99.8% ethanol and stored at -20°C 108 prior to analysis. Ethanol is not normally used to preserve plant material, but is recommended to 109 110 prevent fungal and bacterial growth in bat faeces. The ethanol was evaporated from samples prior to extraction. A total of 95 faecal samples were selected for plant DNA barcoding incorporating 111 approximately equal number of samples from each site: 32 samples from the urban site, 32 112 113 samples from the agricultural site and 31 samples from the secondary forest site. DNA extraction, amplification and sequencing 114 115 We prioritised seeds over pulps to ensure the amplification of DNA and isolated the seeds from the faecal samples. In cases where seeds were not found in the faecal samples, we used the pulps. 116 The seeds and pulps were sent to the Canadian Centre for DNA barcoding (CCDB) for DNA 117 118 extraction, PCR amplification, and Sanger sequencing of two gene regions (rbcL: ~550 bp and 119 ITS2: ~350 bp), following the standard plant protocols of the CCDB (Ivanova and Grainger 2008; 120 Ivanova et al. 2011; Kuzmina and Ivanova 2011a, 2011b).

Plant species identification

We searched GenBank (NCBI 2016) with both the *rbcL* and *ITS2* barcodes to assign taxonomic names to the faecal samples. We prioritised the results of *ITS2* searches over *rbcL* due to the greater taxonomic resolution of this gene fragment (Chen et al. 2010; Kuzmina et al. 2012). We assigned species names based on *ITS2* and *rbcL* matches using a customised set of criteria (Fig. 2). Details of the assignment criterion used for specific samples are given in Online Resource 1. We uploaded the DNA barcodes together with sample metadata to the Barcode of Life Data Systems (BOLD; Ratnasingham and Hebert 2007) under project code VCCBD and the sequences are also available in GenBank under accessions KY080541 to KY080613 and KY080617 to KY080686.

An interaction figure between the bats and detected plants was created to compare the food resource use of *C. brachyotis* at three sampling sites with different land use. The figure was created using the bipartite package (Dormann et al. 2008) in R version 3.3.1. (R Core Team

RESULTS

2016).

Of the 95 faecal samples we analysed, 65 samples (68.4%; seeds=43; pulps=22) produced both *rbcL* and *ITS2* DNA barcodes, 7 samples (7.4%; seeds=5; pulps=2) produced only *ITS2* barcodes, 8 samples (8.4%; seeds=1; pulps=7) produced only *rbcL* barcodes and the remaining 15 samples (15.8%; seeds=2; pulps=13) failed to produce any DNA barcodes (See supplementary file). We discarded two *ITS2* barcodes: one from the urban site due to the short length of usable sequence

(57 bp) and one from secondary forest which was suspected to be a contaminant due to its similarity (96%) to algal sequences (*Chlorella angustoellipsoidea* and *Chloroidium ellipsoideum*). We identified seventeen plant species in the faecal samples using DNA barcoding (Fig. 3; Table 1) of which eight plant species were detected from 26 samples at the urban site, six plant species from 25 samples collected at the agricultural site and seven plant species from 28 samples collected at the secondary forest site. Of the seventeen plant species, we identified nine as native plants and four as exotic plants (Table 1). The status of the remaining four species are unknown as we could not assign them with specific epithets (Table 1). We detected *Ficus fistulosa* at all sampling sites with the highest detection frequency at agricultural and urban sites, and two plant species at two sampling sites with lower detection frequency: *F. lepicarpa* at urban and secondary forest sites, and *Durio zibethinus* at agricultural and secondary forest sites (Fig. 3).

DISCUSSION

Our study suggests that *C. brachyotis* feeds predominantly on pioneer and forest plants. The pioneer plant genus *Ficus* which often dominates regenerating forest (Muscarella and Fleming 2007) emerged as the dominant component of the diet of *C. brachyotis* at all sampling sites with *F. fistulosa* being the most frequently detected plant. Many *Ficus* species including *F. fistulosa*, *F. lepicarpa* and *F. hispida* have multiple fruiting periods throughout the year (Phillipps and Phillipps 2016), making *Ficus* a stable resource compared to more transient species (e.g. *Syzygium jambos* and *Manilkara zapota*) (Corlett 1998; Tan et al. 1998; Fukuda et al. 2009), consequently promoting stable population dynamics in consumers (Tan et al. 2000).

Native forest plants and cultivated plants were detected in faecal samples collected from urban and agriculture sites although we did not observe all the plants at these locations. Seeds belonging to Ficus were found in faecal samples collected from all sites and during our sampling at the urban site, we captured an individual with a *Ficus* fruit in its mouth. This suggests that the fruit bats are moving and depositing seeds away from parent plants, implying the role of fruit bats in seed dispersal. In Thailand, C. brachyotis have been reported to travel up to 14.5 km per day (Bumrungsri 2002) and by transporting seeds across habitats, C. brachyotis could promote plant diversity, particularly in disturbed habitats (i.e. urban and agricultural areas) which often lack seed resources and succession (Hodgkison et al. 2003; McConkey et al. 2012). Exotic plants were detected in the pulps from the faecal samples at all sampling sites. The fairly high detection rate of these exotic plants, particularly P. aduncum and L. chinensis shows that C. brachyotis can exploit novel food resources and potentially could aid invasion of exotic plants through dispersal activities (Muscarella and Fleming 2007). Although we did not visually observe the seeds of exotic plants in the faecal samples nor visually assess the feeding behaviour of C. brachyotis (i.e. carrying fruits away from parent trees to feeding perches), it would be a compelling next step to determine the relative role of the fruit bats in facilitating the succession of native species and/or promoting exotic plant invasions. Our low detection of oil palm (*Elaies guineensis x Elaies oleifera*) in faecal samples collected at agricultural site suggests that the fruit bats are not predominantly feeding on oil palm fruits and their presence in oil palm plantations could be explained by other factors. The diverse diet of C. brachyotis at the oil palm plantation (a monoculture) suggested that the bats may have used the plantation as connecting flyway to travel to forest fragments and agricultural plantations nearby

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which provide more diverse food resources. This is similar to the findings of Heer et al. (2015) which detected a high number of frugivorous bats in rubber-cacao plantations that offered little food resources to the bats, but obviously served as corridors. However, it is also possible we are not detecting oil palm if it is ingested just before they depart from this area though the low detection everywhere suggests this possibility is remote. Our detection of other cultivated plants in faecal samples indicates C. brachyotis feed on other readily available food crops which consequently may lead to conflict between fruit bats and fruit growers. Although the extent of the damage to the food crops caused by C. brachyotis is significantly smaller than that of other larger mammals (i.e. Macaca nemestrina, Arctictis binturong, Cervus timorensis, and Sus barbatus), fruit bats are often killed in large numbers as they are generally of lower concern to the wildlife authorities (Fujita and Tuttle 1991; Aziz et al. 2016). We detected plants (i.e. Ficus fistulosa, Szygium jambos, and Pellacalyx saccardianus) which have previously been reported in diet studies of C. brachyotis conducted at secondary forest and urban areas (Phua and Corlett, 1989; Tan et al. 1998). However, we also failed to detect many plants which were reported to be seasonally dominant in the diet of the fruit bats, most likely due to our short sampling period. Nevertheless, our use of DNA barcoding detected cultivated plants (i.e. Parkia roxburghii, Elaies guineensis x Elaies oleifera, and Coccinia grandis) and pioneer plants (i.e. Ficus hispida and F. lepicarpa) which have not been reported in other studies of the diet of C. brachyotis (Phua and Corlett, 1989; Tan et al. 1998; Hodgkison et al. 2004). The advantage of using DNA barcoding to identify the diet of *C. brachyotis* is that we were able to assign species names to most of the seeds and digested plant pulp in the faeces. With DNA barcoding, most of the seeds were assigned with the species name Ficus fistulosa which also has

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been reported by Phua and Corlett (1989) and Tan et al. (1998) as the most common Ficus eaten by C. brachyotis at secondary forest and urban areas. Seeds belonging to Ficus can be easily assigned to this plant genus based on the morphology of the seeds. However, assigning Ficus seeds accurately to a species based on the morphology of the seeds is often time-consuming and requires high level of plant taxonomic expertise. Phua and Corlett (1989) failed to assign species name to six types of *Ficus* remains due to the difficulty in identifying the remnants of the seeds and fruits while Hodgkison et al. (2004) germinated the seeds collected from faeces of bats for species identification based on the morphology of the seedlings. However, our reliance on existing databases and local floral records leaves these names as provisional. We assigned most of the ITS2 sequences with species names as the region is able to distinguish closely related species within same genus when comprehensive reference libraries are available (Braukmann et al. 2017). However, the region produces some ambiguous results in rapidly radiating groups (e.g. Ficus) and in our case, local botanical records were used to refine these cases. We observed that the ITS2 region detected fewer plant families compared to rbcL. In contrast, most of the *rbcL* sequences matched to sequences in GenBank recorded under multiple species names with 100% similarity. High-throughput sequencing (HTS) could be utilized for future diet studies of fruit bats, which may help distinguish mixed signals in individuals consuming multiple species, although the smaller read length of HTS platforms may compromise some plant identifications. We detected plants (i.e. *Elaies guineensis* x *Elaies oleifera* and *Coccinia grandis*) with seeds that are too large to be ingested by C. brachyotis and which consequently are not observed morphologically in the faeces. Although the fruit bats may not be able to disperse large seeds

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through defecation, C. brachyotis may still serve as important seed disperser by carrying the heavy fruits with large seeds to feeding perches away from parent trees (Funakoshi and Zubaid 1997). Therefore, our findings highlight the importance of using DNA barcoding in dietary studies of fruit bats, as the reliance on morphological identification of seeds in the faeces may overlook plants with large seeds where only pulp is present and consequently overlook the potential seed dispersal role of the fruit bats. We preferentially selected seeds rather than fruit pulp for sequencing. If a bat had consumed a large fruit (and dropped the large seed) along with a small fruit (and swallowed the small seeds), it may potentially cause a bias in the detection of small seeded plants. However, the gut passage time of most fruit bats is fast enough that we do not frequently see multiple fruit types in a sample (E Clare personal observation) and thus the effect of the bias is likely minimal. One limitation of using DNA barcoding to identify the species origin of plant pulp is that we cannot determine which part of the plant the fruit bats are feeding on. For example, the most important pollinator of economically important Durio zibethinus is Eonycteris spelaea which feeds on nectar (Bumrungsri et al. 2009), whereas C. bracyotis is reported to feed on the flowers (Funakoshi and Zubaid 1997). Although we detected D. zibethinus in the diet of C. brachyotis, we could not determine whether the fruit bats feed on nectar and consequently pollinate the economically important crops, or are consuming the fruits and/or flowers which would inhibit the development of the crops.

CONCLUSION

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The diet of C. brachyotis at secondary forest, urban and agricultural sites was compared using DNA barcoding (i.e. Sanger sequencing). The high detection of *Ficus* seeds in the faeces of *C*. brachyotis indicates that the bats rely heavily on this native food source in all habitats but the fairly high detection of exotic and cultivated plants in the faeces suggests that C. brachyotis is flexible and can exploit exotic and cultivated plants as novel food resource. The diverse diet of C. brachyotis at the oil palm plantation indicated that the fruit bats are not predominantly feeding on oil palm fruits but cultivated plants nearby the plantation. Together these observations suggest an interesting dual role of C. brachyotis in dispersing (i) native pioneer plants which aid in forest regeneration and (ii) non-native plants which potentially facilitate their invasion, consequently suggesting a research avenue that deserves further investigation. The use of DNA barcoding in this study enabled the detection of plant species that had not been reported in previous diet studies of C. brachyotis but does not provide information regarding which part of the plant was consumed by the bats. Nevertheless, this study demonstrated the utility of DNA barcoding in dietary studies of frugivorous bats and the extent to which C. brachyotis is capable of adapting to changing landscapes and plant resources.

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281 CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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382	SUPPORTING INFORMATION
383	Additional supporting information can be found in the online version of this article.
384	Online Resource 1. Criteria used to assign species names to DNA barcodes.
385	DATA ACCESSIBILITY
386	Morphological data and raw sequence data are provided in BOLD under project code VCCBD
387	and available in GenBank under accessions KY080541 to KY080613 and KY080617 to
388	KY080686.
389	

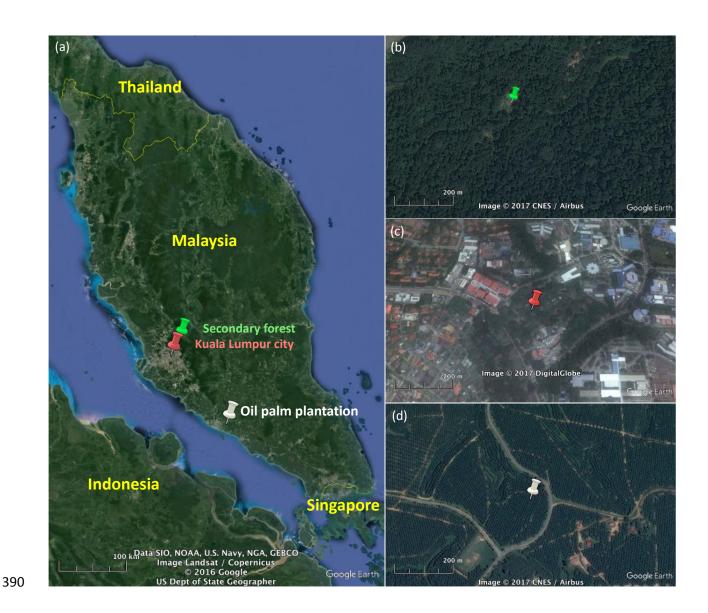


Fig. 1 The sampling location in Peninsular Malaysia. (a) The map of Peninsular Malaysia. (b)

The sampling location at secondary forest. (c) The sampling location at urban area. (c) The sampling location at oil palm plantation.

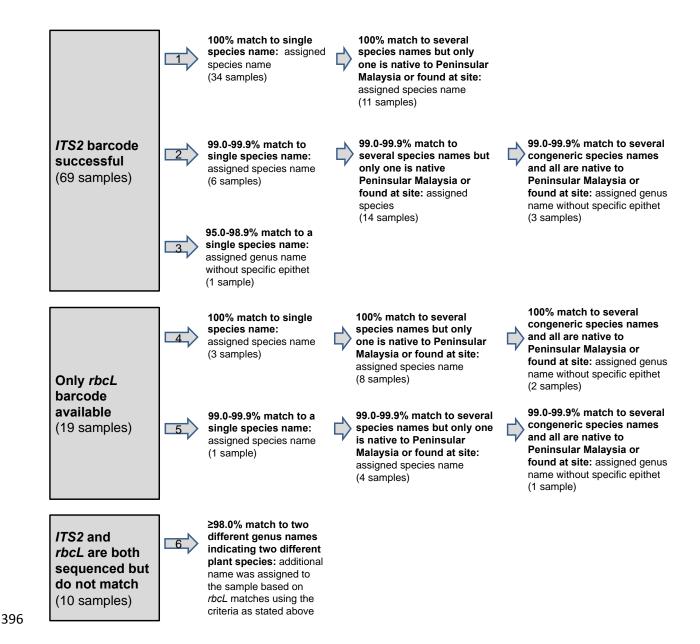


Fig. 2 Criteria used in assigning taxonomic names to the plant DNA barcodes based on matches returned by BLAST searches on Genbank, NCBI database

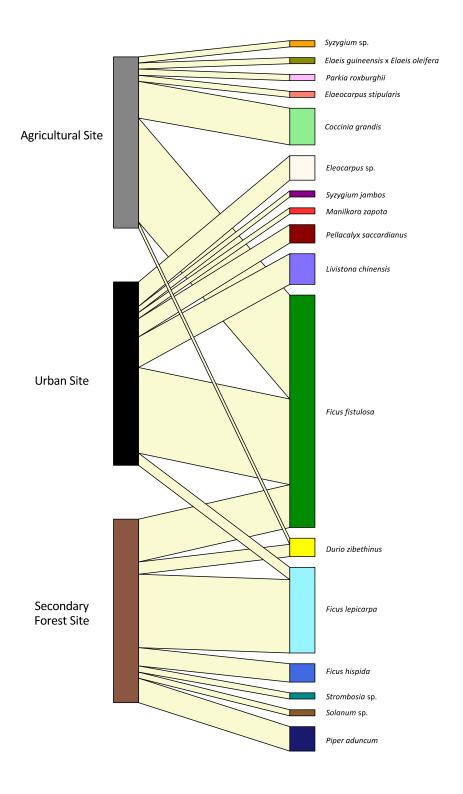


Fig. 3 The interaction between *C. brachyotis* and plant species detected from faecal samples collected at three sites in Peninsular Malaysia. The width of the interaction bar corresponds to the number of fruit bats and occurrence of plants in the faeces of fruit bats.

Table 1. List of plants consumed by *C. brachyotis* in Peninsular Malaysia and Singapore. Phua & Corlett (1989) reported 21 plant species through observation of feeding behaviour of *C. brachyotis* at the botanical garden in Singapore. Tan *et al.* (1998) reported 53 plant species through morphological identification of the plant remnants in the masticated pellets of *C. brachyotis* at secondary forests in Peninsular Malaysia. Hodgkison *et al.* (2004) reported fifteen plant species through morphological identification of the plant remnants in the faeces, on the bodies and under the roosts of *C. brachyotis* at primary forest in Peninsular Malaysia.

Family	Species	Status ^a	Phua & Corlett 1989	Tan et al. 1998	Hodgkison et al. 2004	This study
Moraceae	Artocarpus fulvicortex	N		X		
	Artocarpus maingayi	N		X		
	Ficus fistulosa	N	X	X		X
	Ficus benjamina	N		X		
	Ficus globosa	N			X	
	Ficus hispida	N				X
	Ficus lepicarpa	N				X
	Ficus magnoliifolia	N			X	
	Ficus religiosa	Е		X		
	Ficus scortechinii	N			X	
	Ficus (Unidentified)			X		
Leguminosae	Bauhinia purpurea	Е		X		
	Cassia fistula	Е		X		
	Parkia roxburghii	N				X
	Peltophorum pterocarpum	N		X		
	Senna spectabilis	Е		X		
	Erythrina subumbrans	N		X		
	Erythrina variegata	N		X		

	Erythrina fusca	Е		X		
	Erythrina (Unidentified)			X		
Sapotaceae	Manilkara zapota	Е		X		X
	Mimusops elengi	N		X		
	Palaquium clarkeanum	N		X		
	Palaquium gutta	N	X	X		
	Palaquium obovatum	N	X	X	X	
	Payena selangorica	N		X		
	Payena lucida	Е		X	X	
	Payena maingayi	N		X		
	Pouteria malaccensis	N		X		
Myrtaceae	Psidium guajava	Е	X	X		
	Syzygium jambos	N	X	X		X
	Syzygium chloranthum	N			X	
	Syzygium grande	N	X	X		
	Syzygium aqueum	N		X		
	Syzygium malaccense	N	X	X		
	Syzygium lineatum	N	X			
	Syzygium (Unidentified)					X
	Eugenia (Unidentified)			X	X	
Arecaceae	Dypsis lutescens	Е		X		
	Elaies guineensis x Elaies oleifera	Е				X
	Ptychosperma macarthurii	Е		X		
	Roystonea regia	Е		X		

	Saribus rotundifolius	Е		X		
	Licuala grandis	Е		X		
	Livistona chinensis	Е		X		X
Annonaceae	Annona squamosa	Е		X		
	Cyathocalyx scortechinii	N			X	
	Polyalthia longifolia	Е		X		
Anacardiaceae	Campnosperma auriculatum	N	X			
	Mangifera indica	Е		X		
Pentaphylacaceae	Adinandra dumosa	N	X			
	Adinandra sarosanthera	N			X	
Elaeocarpaceae	Elaeocarpus stipularis	N		X	X	X
	Elaeocarpus (Unidentified)			X		X
Malvaceae	Grewia tomentosa	N		X		
	Durio zibethinus	N				X
Clusiaceae	Calophyllum inophyllum	N	X	X		
Combretaceae	Terminalia catappa	N	X	X		
Cucurbitaceae	Coccinia grandis	N				X
Euphorbiaceae	Hevea brasiliensis	Е		X		
Gentianaceae	Fagraea fragrans	N	X	X		
Lamiaceae	Vitex pinnata	N	X			
Melastomataceae	Pternandra echinata	N		X	X	
Muntingiaceae	Muntingia calabura	Е	X	X		
Olacaceae	Strombosia javanica	N			X	

	Strombosia					X
	(Unidentified)					
Piperaceae	Piper aduncum	Е		X		X
Podocarpaceae	Podocarpus rumphii	N	X			
Rhizophoraceae	Pellacalyx saccardianus	N	X	X	X	X
Rosaceae	Prunus polystachya	N			X	
Rubiaceae	Nauclea officinalis	N			X	
Salicaceae	Flacourtia inermis	Е		X		
Sapindaceae	Nephelium malaiense	N	X	X		
Urticaceae	Cecropia peltata	Е	X			
Ebenaceae	Diospyros (Unidentified)		X	X		
Musaceae	Musa (Unidentified)		X	X		
Solanaceae	Solanum (Unidentified)					X

a = Status of plants (N = native, E = exotic)