GENETIC VARIABILITY, DIET METABARCODING, AND CONSERVATION OF COLOBINE PRIMATES IN VIETNAM

by

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Genetic Variability, Diet Metabarcoding, and Conservation of Colobine Primates in Vietnam

Thesis directed by Professor Herbert H. Covert

This dissertation examines the genetic variability and diet of three colobine species across six sites in Vietnam: the endangered black-shanked douc (*Pygathrix nigripes*, BSD) in Ta Kou Nature Reserve, Cat Tien National Park, Nui Chua National Park, and Hon Heo Mountain; endangered Indochinese silvered langur (*Trachypithecus germaini*, ISL) in Kien Luong Karst Area (specifically Chua Hang, Khoe La, Lo Coc and Mo So hills); and critically endangered Tonkin snub-nosed monkey (*Rhinopithecus avunculus*, TSNM) in Khau Ca Area. A total of 395 fecal samples were collected (July 2012-October 2014) and genomic DNA was extracted. This research provides the first information on their mitochondrial hypervariable region I variability and also pioneers the characterization of their diet using DNA metabarcoding in association with next-generation sequencing.

BSDs exhibit high variability but no gene flow between populations. Similarly, ISLs showed high variability but only the subpopulation in Khoe La, the site under mining disturbance, retains most of the remaining genetic diversity in the species. Zero mitochondrial variability was found in TSNMs, the lowest ever reported for primate species in the wild.

Diet sequences of colobines were matched to plant databases for identifications. Forty plant families were recorded for BSDs, including new records from 18 families, 15 genera and 13 species. There was little overlap in their diet with only six taxa found across four populations. They were also selective by feeding on less abundant species. Twenty-five families were recorded for ISLs, including new records from nine families, 18 genera and 14 species. Moraceae dominated their diet as retrieved from fecal samples, and was also the top family as revealed by field observations, demonstrating the significance of this plant family

in their diet. Eighteen families were identified for TSNMs, including new records from three families, five genera and three species. The dominant taxon belonged to *Polyalthia* (Annonaceae) and they were also the dominant genus and family respectively within Khau Ca Area.

This research highlights the importance of using genetic methods to complement field observations so as to better understand population viability and dietary profiles in order to identify priority actions for the conservation of colobine primates.

(350 words)

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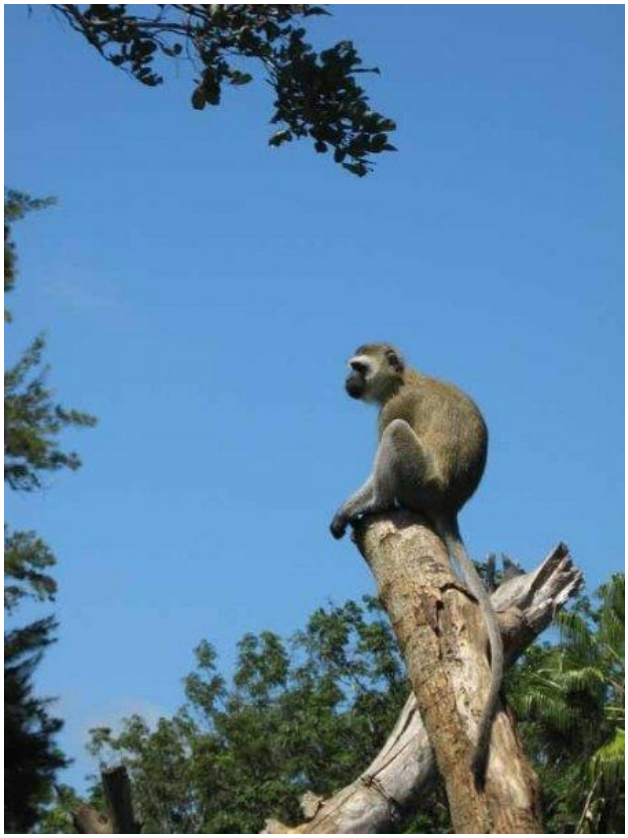

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2. Srivathsan A., **Ang A.**, Vogler A.P. and Meier R., 2016. Fecal metagenomics for the simultaneous assessment of diet, parasites, and population genetics of an understudied primate. Frontiers in Zoology 13, 17 .....(Chapter 4)
3. Hoang Minh Duc, Covert H., Moody J. and **Ang A.**, in prep. *Trachypithecus germaini*. The IUCN Red List of Threatened Species (Chapter 2)

INTRODUCTION

1.1. Research Aims

The aim of this dissertation is three-fold: first, to examine the genetic variability of populations of highly threatened species of colobine monkeys in Vietnam; second, to describe their dietary profiles using DNA metabarcoding in association with next-generation sequencing (NGS) techniques; and lastly, to discuss implications of the results and to suggest priority actions that could contribute to their conservation in Vietnam. Specifically, this research investigates the mitochondrial genetic variability and describes the plant diet of the 1) black-shanked doucs (*Pygathrix nigripes*; endangered) in Ta Kou Nature Reserve, Cat Tien National Park, Nui Chua National Park, and Hon Heo Mountain; 2) Indochinese silvered langurs (*Trachypithecus germaini*; endangered) in Chua Hang, Khoe La, Lo Coc, and Mo So hills in the Kien Luong Karst Area; and 3) Tonkin snub-nosed monkeys (*Rhinopithecus avunculus*, critically endangered) in the Khau Ca Area.

1.2. Colobine Monkeys

Old World monkeys (Family: Cercopithecidae) are a diverse radiation of primates found in Asia, Africa, and Gibraltar with two extant subfamilies: Colobinae, which includes colobus monkeys, langurs and odd-nosed monkeys, and Cercopithecinae, which includes macaques, baboons, mangabeys, mandrills, and guenons. Colobine monkeys are characterized by having multi-chambered stomachs with enlarged foregut that contains a vast and diverse array of microflora needed to process and ferment plant material (Bauchop & Martucci 1968; Strasser & Delson 1987). They also have molars with well-developed shear crests (Fleagle 2013) adapted for shearing tough food items such as leaves. Thus they exhibit an enhanced ability to consume foliage, but they also take in significant amount of other plant parts such as fruits and seeds.

Colobine monkeys consist of two clades, one in Africa and one in Asia which diverged between 10 and 13 million years ago in Africa (Delson 1994; Goodman *et al.* 1998). Asian colobines (langurs and odd-nosed monkeys; 55 species) are more speciose than their African cousins (colobus monkeys; 23 species) and exhibit a suite of morphological, behavioral, and ecological variation with a large geographic distribution in Asia. Currently 55 species are recognized in seven genera (*Nasalis, Presbytis, Pygathrix, Rhinopithecus, Semnopithecus, Simias,* and *Trachypithecus*; Mittermeier *et al.* 2013). Asian colobines are found in a multitude of habitats from tropical and subtropical forests (e.g. Javan leaf monkey, *Presbytis comata*; Tonkin snub-nosed monkey, *R. avunculus*), swamp forests (e.g. Raffles' banded langur, *Presbytis femoralis*; proboscis monkey, *N. larvatus*), limestone karst forests (Delacour's langur, *T. delacouri*; François' langur, *T. francoisi*), along coastlines (e.g. silvered leaf monkey, *T. cristatus*), in mountains as high as 4,700 m in elevation (e.g. black snub-nosed monkey, *R. bieti*), and in human-dominated landscapes (Bengal Hanuman langur, *Semnopithecus entellus*, where some of the populations live in the cities; Mitra & Molur 2008).

The social structure of Asian colobines shows a similar diversity. The most common social unit consists of one-male and several females with their young but occasionally, these uni-male groups come together to form multimale-multifemale units such as in the dusky leaf monkey (*T. obscurus*), red-shanked douc (*Pygathrix nemaeus*), Chamba sacred langur (*Semnopithecus ajax*), and proboscis monkey (Curtin 1980; MacKinnon & MacKinnon 1980; Bennett & Sebastian 1988; Lippold 1998; Minhas *et al.* 2012). In the snub-nosed species (*Rhinopithecus* spp.), these multimale-multifemale units can often band together to form a large group of more than 700 individuals (see Bleisch *et al.* 2008). On the other hand, the Mentawai langur (*Presbytis potenziani*) exhibits a rather distinctive social organization

among Old World monkeys in forming monogamous pairs and performing duets between males and females (Tilson & Tenaza 1976).

Sexual dimorphism in body size is apparent in odd-nosed colobines (*Nasalis*, *Pygathrix*, *Rhinopithecus*, and *Simias*) where adult males can weigh up to 1.5-2x as much as adult females (e.g. proboscis monkey, males 20.4kg and females 9.8kg; golden snub-nosed monkey, *R. roxellana*, males 17.9kg and females 11.6kg) but in many of the langurs (e.g. *Presbytis* and *Trachypithecus*) body size alone is insufficient to tell apart males and females and they are difficult to distinguish especially in the field (e.g. maroon leaf monkeys, *P. rubicunda*, males 6.3kg and females 6.2kg) (see Fleagle 2013).

Asian colobines are also a highly threatened group of primates with 75% of the species currently listed as threatened (Mittermeier *et al.* 2013): nine species are considered critically endangered, 23 species endangered, and nine species vulnerable. Tropical forest destruction, habitat conversion, hunting, and pet trade are some of the major problems driving many populations to extinction. In Borneo, all species of *Presbytis* langurs, such as the Hose's langur (*P. hosei*), white-fronted surili (*P. frontata*) and Sarawak's surili (*P. chrysomelas*) are severely threatened by hunting for their bezoar stones for traditional medicine (e.g. Nijman 2005; Setiawan *et al.* 2009; Ehlers Smith 2014). The pig-tailed langur, or the simakobu monkey (*Simias concolor*) is critically endangered (Whittaker & Mittermeier 2008) and endemic to the Mentawai islands in Indonesia. In just 10 years, its population density was estimated to have declined by 73-90% due to hunting pressures which was intensified by the logging and conversion to palm oil plantations of their natural habitats, making it easier for hunters to access the animals (Paciulli 2004; Whittaker *et al.* 2006).

1.3. Genetic Variability

Loss, fragmentation, and degradation of habitat reduces the distribution of many species to a number of small and/or isolated populations across their ranges. These population

fragments have a heightened risk of loss from the effects of genetic deterioration, disease outbreak, extreme weather and other catastrophic events. An understanding of their viability over the long-term is fundamental (Mittermeier *et al.* 1999a), and securing a future for these threatened species will require targeted action in a number of areas such as identifying remaining population sizes and composition, distribution, habitat use and requirements, and the degree of genetic variability left.

Genetic variability can be defined as the variation in alleles of genes in the gene pool of a population or a species with mutations and sexual reproduction as the main causes for genetic variability, while genetic diversity can be defined as the sum total of genetic information, contained in the genes of individuals of plants, animals, and microorganisms (McNeely *et al.* 1990). Simply, genetic variability provides the raw material for natural selection while genetic diversity forms the baseline of biodiversity. However, many studies use genetic variability and genetic diversity interchangeably to refer to the amount of genetic variation in a population or a species (e.g. Hoda & Marsan 2012; Larson 2012).

In addition to factors including population size, reproductive output, environmental changes, and human activities, genetic variability within a species is regarded as an important factor for the long-term survival of species and contribute to the assessment of a species' conservation status (Dietz *et al.* 2000; Di Fiore 2003; Höeglund 2009). Small populations rapidly lose genetic variability through genetic drift and inbreeding (Lande & Barrowclough 1987). Diminished genetic variability within populations, or loss of distinct populations, reduces the opportunity for adaptive responses to geographically varying local conditions (Sherwin & Moritz 2000), and may result in reduced reproduction or survival and thereby reduces the viability of the population (Nei *et al.* 1975; Madsen *et al.* 1996).

Mating between related individuals results in inbreeding depression, i.e. a decline in fitness, where the relative performance such as in birth weight, survival, and reproduction of

the resulting inbred progeny is lower compared to progeny produced from mating between unrelated individuals within a population (e.g. Crnokrak & Roff 1999; Keller & Waller 2002). In captive groups of rhesus macaques (*Macaca mulatta*), Smith (1986) showed that inbred offspring (n=101) had lower birth weight compared to non-inbred offspring (n=94). In captive groups of Goeldii's marmosets (*Callimico goeldii*), inbred conceptions (n=111) resulted in 46% offspring death before one month of age while non-inbred conceptions (n=679) resulted in 24% mortality (Lacy *et al.* 1993). Similarly, in wild populations of yellow baboons (*Papio cynocephalus*), inbred conceptions (n=3) resulted in 100% offspring death before 30 days while non-inbred conceptions (n=140) resulted in 19% infant mortality (Alberts & Altmann 1995). In semi-free ranging mandrills (*Mandrillus sphinx*), more heterozygous females produced more offspring than less heterozygous individuals (n=52), and more heterozygous alpha males sired more offspring (n=9) (Charpentier *et al.* 2005a). For semi-free ranging sun-tailed monkeys (*Cercopithecus solatus*), interbirth interval following births of inbred offspring (n=3) was longer than of non-inbred offspring (n=6) (Charpentier *et al.* 2005b). Decreased genetic variability may also be associated with increased susceptibility to parasites (e.g. Charpentier *et al.* 2008). In a study of 73 ring-tailed lemurs (*Lemur catta*), it was found that more homozygous adult individuals were more susceptible to infection by warbles (*Cuterebra* larvae) than their heterozygous peers (Charpentier *et al.* 2008).

Population genetic models predict that the increased homozygosity resulting from inbreeding will expose recessive deleterious alleles to selection such that further inbreeding will become harmless, after purging of the genetic load (Waller 1993). Indeed, some studies found no inbreeding depression in their studied species, indicating the possibility that such purging may have happened (e.g. Wisely *et al.* 2002; Thünken *et al.* 2007). The black-footed ferret (*Mustela nigripes*) is an endangered North American carnivoran that underwent a well-

documented population bottleneck during the 1980s (Belant *et al.* 2015). The species declined to near extinction by the late 1970s, primarily as a consequence of simultaneous epizootics of canine distemper and sylvatic plague (Belant *et al.* 2015). Eighteen individuals of the last 40 adults in the wild were then captured for breeding; by 1986, no animals were known in the wild and only seven adults bred in captivity contributed to the present gene pool. After a captive breeding program, it was reintroduced into eight western states and Mexico from 1991 to 2008 (Belant *et al.* 2015). At present, it is considered self-sustaining at only three sites (one in Wyoming and two in South Dakota). Genetic analyses of museum specimens from the pre-bottleneck 1891 Kansas population, during bottleneck 1972 South Dakota population, and the 1985 pre- and during bottleneck Wyoming populations showed that genetic diversity in black-footed ferrets was greatest in the large undisturbed population of Kansas (Wisely *et al.* 2002). When the Wyoming population declined to the seven genetic founders of the captive group, all three measures of genetic variability (expected heterozygosity, percent of polymorphic loci, and number of alleles per locus averaged across loci) decreased below historic levels (Wisely *et al.* 2002). Despite this, litter sizes in wild-born individuals did not differ before versus after the bottleneck in Wyoming populations, suggesting that at least some components of fitness were not correlated with the observed decline in genetic diversity (Wisely *et al.* 2002). The authors attributed the unchanged fitness to several possible explanations. Because the Wyoming subpopulation was likely small and isolated during the Holocene, the homozygous population may have been able to purge deleterious alleles, accounting for the lack of inbreeding depression in the post-bottleneck population (Lande 1988). Under this same sequence of continuous isolation and small population size, fitness could have been reduced by the fixation of deleterious alleles, lowering litter size before the population's discovery in 1981 (Wisely *et al.* 2002). Nevertheless, even if lethal mutations can be quickly purged via inbreeding, the large fitness

costs of this purging may affect population viability (Barret & Charlesworth 1991). Moreover, mutations that are only slightly deleterious are difficult to eliminate and are the principle cause of inbreeding depression (e.g. Wang 2000).

A review of empirical data on inbreeding depression from 14 studies on wild, free-ranging, and captive non-human primate populations between 1979 and 2006 also showed that inbreeding may not affect all fitness traits equally in different populations of the same species or even in the same population (Charpentier *et al.* 2007). The intensity of inbreeding depression may vary depending on life-history stage, trait measured, or environmental conditions (Keller & Waller 2002). For example, in captive rhesus macaques, even though inbred offspring showed lower birth weight, inbreeding depression did not occur for yearling mortality or female fertility (Smith 1986). Similarly, in semi-free ranging mandrills, although females and alpha males that were more inbred experienced reduced reproduction relative to those that were not inbred, the reproductive success of subordinate males was not affected by inbreeding depression (Charpentier *et al.* 2005a)

Nonetheless, small and fragmented populations with limited sex-biased dispersal, perhaps due to deforestation or other anthropogenic disturbance will experience population subdivision and hence potentially severe inbreeding depression. Fragmentation often reduces the number of breeding individuals within a population while reducing gene flow between populations. The genetic structure based on microsatellite loci and the dispersal patterns of two populations of black howler monkeys (*Alouatta caraya*) were compared to examine genetic variability and effects of habitat fragmentation on gene flow by Oklander *et al.* (2010). These researchers noted that groups of howler monkeys which experienced a reduction in the ability to disperse in fragmented forests showed greater genetic clustering and adult females are more genetically closely related than those groups in continuous

forests. Studies on inbreeding depression are thus highly imperative, particularly in threatened populations facing habitat destruction and fragmentation.

Overall, based on current knowledge of population genetics, we may have general expectations on how factors such as population size and habitat size affect genetic variability, e.g. it is expected that endangered primates would exhibit low levels of genetic variability due to elevated genetic drift in their small populations, and show a strong correspondence between genetic and geographic distance due to disruption of gene flow between forests by habitat fragmentation. However, published literature on primate population genetics over the last decade as summarized above demonstrates that it is no simple relationship.

Assessment of genetic variability is ideally based on mitochondrial and nuclear markers, but this is difficult when working with fecal DNA for poorly studied species that lack species-specific primers. As much as fecal samples provide low-quality DNA, it is the best alternative source of DNA given that it is extremely risky and difficult to acquire fresh blood or hair samples from unhabituated and rare arboreal primates. Mitochondrial loci can often still be assessed, but nuclear microsatellite primers are usually not available. Microsatellite primers for closely related species are the next best choice, but they often amplify poorly and/or the amplified sequences lack variability. This is why the initial assessment of genetic variability for nonmodel species usually still starts with mitochondrial markers (see Leese & Held 2011). The mitochondrial hypervariable region I (HV-I) of the displacement loop (d-*loop*) is often used to evaluate the level of genetic diversity in primate populations, especially in colobine primates (e.g. Li *et al.* 2007; Liu *et al.* 2007; McDonald & Hamilton 2010; Munshi-South & Bernard 2011; Yang *et al.* 2012; Ang *et al.* 2016; see **Chapter 3 on Genetic Variability of Colobine Primates**).

1.4. Diet

Feeding is a fundamental aspect of an animal's ecology (Hohmann *et al.* 2007; Lambert 2011) and one of the greatest influences in primate life histories, abundance and demography (Leigh 1994; Balko & Underwood 2005). Examining the interactions between primates and their habitats allows us to better understand their feeding ecology. The botanical compositions of their habitat, phenology of the plants (thus availability of plant parts), and chemical and mechanical properties of plant parts (e.g. toughness and stiffness) are important factors affecting the selectivity of plant species by primates (e.g. van Schaik *et al.* 1993; Wright *et al.* 2008; Huang *et al.* 2010; O'Brien 2014) such as colobines with a plant-dominated diet.

Leaves are often defended by toxins such as phenols and digestion-inhibiting materials such as tannins and fibers so that even though they are ubiquitous in the forest, folivorous colobines must be selective in the plant parts and species they consume (Kirkpatrick 2011). Generally, colobine monkeys choose young leaves over mature leaves, e.g. Mentawai langur (Fuentes 1996), Delacour's langur (Workman 2010), and black-and-white snub-nosed monkey (Grueter *et al.* 2009a). Huang *et al.* (2010) showed that black-and-white snub-nosed monkeys preferred leaves with lower fiber content and lower toughness while no significant difference was found for crude protein between preferred and non-preferred leaves. However, this is not always the case. O'Brien (2014) showed that the black-shanked doucs (*Pygathrix nigripes*) in Cat Tien National Park of Vietnam chose leaves with not only higher protein but also higher fiber, which contradicts a long-standing understanding that leaves with lower amount of fiber will always be selected (e.g. Yeager *et al.* 1997). This could be related to the gut morphology of doucs in general which enables them to break down more fibers than other colobines (Caton 1998; Wright *et al.* 2008).

Not all plant parts are available year-round especially in seasonal habitats, forcing colobines to switch dietary preference during different months of the year. For instance,

capped langurs (*T. pileatus*) subsisted on mature leaves during the dry season (comprising 80% of their diet from November to March), and consumed seasonal items like new leaves and fruits when they became available during the hot dry (20%-60% diet from March to May) and the monsoon (50% diet from May to September) periods respectively (Stanford 1991). Similarly, western purple-faced langurs (*Semnopithecus vetulus nestor*) maintained a consistently high proportion of seasonal foods in their diet by exploiting a large number of species with asynchronous phenological cycles, particularly for fruits (Dela 2007). These data suggest that the feeding strategy of some colobines is adapted to cope with seasonal food availability. In addition to leaves, some colobine monkeys have also been documented to consume large amounts of other plant parts such as seeds. Forty percent of the diet of the black-shanked doucs in Seima Biodiversity Conservation Area, Cambodia consisted of seeds (Rawson 2009) and in the maroon langurs in Kalimantan, Indonesian Borneo, seeds composed 76.4% of their mean annual diet (Ehlers Smith *et al.* 2013).

Characterizing the diet of an endangered primate becomes essential for structuring effective management plans for its conservation. Conservation measures such as choosing areas for protection or species to be planted for remediation of degraded habitat can be guided if food plants are known. Obtaining this information, however, is challenging for primates because diet varies throughout the year and geographical range (Barnett 1995). Moreover, the total number of food plant species consumed by primates can vary tremendously. While Phayre's leaf monkeys (*T. phayrei*) fed on only 18 plant species (Gupta & Kumar 1994), Hoang Minh Duc *et al.* (2009) recorded 152 food plants for black-shanked doucs. Both studies included at least seven months of fieldwork (excluding habituation time) to collect dietary information. Furthermore, following rare and unhabituated primates to identify food items through direct observation in less accessible locations or challenging terrains is difficult. Therefore, an extended field study period is needed before a reasonably

comprehensive dietary profile of an endangered species can be obtained. Alternatively, molecular genetic methods can be employed to complement observational study so that food items can be identified in a relatively shorter period of time. The emergence of next-generation sequencing (NGS) technologies in association with DNA metabarcoding has recently made possible the performance of rapid diet analysis by identifying DNA sequences of consumed items from fecal material by directly sequencing in parallel thousands of short DNA barcodes amplified using universal primers (see Taberlet *et al.* 2012).

1.5. Primate Conservation in Vietnam

Vietnam and a number of other Southeast Asian countries including Lao PDR, Cambodia, Thailand, and Myanmar belong to the Indo-Burma biodiversity hotspot which houses a rich diversity of endemic species (Mittermeier *et al.* 1999b, 2004). The rich biota of this region is also exemplified by the recent discoveries or rediscoveries of several large mammalian species such as the grey-shanked douc, Burmese snub-nosed monkey, saola, and the large-antlered muntjac. However, this region is also characterized by widespread threats to wildlife and ecosystems.

Continuing the trends of past millennia, Vietnam's population grew exponentially during the 20th century from 15.6 million people in 1920s to over 95 million today (Central Intelligence Agency 2016). As the country's population increases, so do demands on natural resources. With more than 50% of the land under intensive agriculture, wildlife and ecosystems are under constant and growing threats (Sterling *et al.* 2006). Habitat destruction and conversion for development is one of the most extensive threats to local biodiversity. Timber extraction and hunting pressures are also causing significant population declines to a wide range of wildlife taxa. At present, approximately 40% of total land area in Vietnam is forested, of which 17% consists of primary forests (Food & Agriculture Organization 2010).

Vietnam has the highest number of primate taxa in mainland Southeast Asia with 25 taxa (Blair *et al.* 2011). The primates of Vietnam (Table 1.1.) are one of the top global priorities for primate conservation. Nearly 90% of Vietnamese primate species are listed as vulnerable, endangered, or critically endangered, and more than 60% are severely threatened by hunting for food and medicinal products (Ripple *et al.* 2016). Until recently, five taxa had been consistently included among the world's top 25 most endangered primates (Mittermeier *et al.* 2012). In the 2014-2016 list, the Tonkin snub-nosed monkey (*Rhinopithecus avunculus*), Cat Ba langur (*Trachypithecus poliocephalus poliocephalus*), and Delacour's langur (*T. delacouri*), which are colobine primates and also endemic to Vietnam, are among the top 25 (Schwitzer *et al.* 2015). Additionally, all 11 species of colobines are either endangered or critically endangered.

Table 1.1. Primates in Vietnam

Taxa		Common name	Conservation status
STREPSIRRHINI			
Lorisidae			
	Nycticebus pygmaeus	Pygmy slow loris	VU
	N. bengalensis	Bengal slow loris	VU
HAPLORRHINI			
Cercopithecidae			
Colobinae			
	Trachypithecus crepusculus[1]	Grey langur	EN
	*T. delacouri**	Delacour's langur	CR
	T. francoisi	Francois' langur	EN
	T. germaini	Indochinese silvered langur	EN
	T. margarita[2]	Annamese silvered langur	EN
	T. hatinhensis	Hatinh langur	VU
	*T. poliocephalus poliocephalus**	Cat Ba langur	CR
	Pygathrix cinerea[#]	Grey-shanked douc	CR
	P. nemaeus	Red-shanked douc	EN
	P. nigripes	Black-shanked douc	EN
	*Rhinopithecus avunculus**	Tonkin snub-nosed monkey	CR
Cercopithecinae			
	Macaca arctoides	Stump-tailed macaque	VU
	M. assamensis	Assamese macaque	NT
	M. fascicularis	Long-tailed macaque	LC
	M. fascicularis condorensis	Con Song long-tailed macaque	VU
	M. leonina	Northern pig-tailed macaque	VU
	M. mulatta	Rhesus macaque	LC
Hylobatidae			
	Nomascus annamensis[3]	Northern buff-cheeked gibbon	NE
	N. concolor	Black-crested gibbon	CR
	N. gabriellae	Yellow-cheeked crested gibbon	EN
	N. leucogenys	Northern white-cheeked gibbon	CR
	N. nasutus[#]	Cao-vit crested gibbon	CR
	N. siki	Southern white-cheeked gibbon	EN

[1]Included in *Trachypithecus phayrei* by IUCN but recently elevated to full species status (Liedigk *et al.* 2009); [2]Included in *Trachypithecus germaini* by IUCN but recently elevated to full species status (Hoang Minh Duc *et al.* 2012); [3]Recently named species yet to be listed by IUCN (Van Ngoc Thinh *et al.* 2010); *Listed among top 25 most endangered primates (Schwitzer *et al.* 2015); #Formerly listed among top 25 most endangered primates; Conservation status according to the IUCN Red List of Threatened Species: CR = critically endangered, EN = endangered, VU = vulnerable, NT = near threatened, LC = least concern, NE = not evaluated

Over the past few decades, local government agencies and conservation institutions have been actively working to protect and manage biodiversity and habitats (see Le Khac Quyet 2014), and have been welcoming to international organizations and scientists to contribute to the growing field of primate research in the country. We see a rise in the number of graduate research theses and projects on primate ecology, behavior, gut microbiome, and conservation, notably of colobines (e.g. Hoang Minh Duc 2007 and O'Brien 2014: Ecology and conservation of black-shanked doucs; Ha Thang Long 2009: Behavior of grey-shanked doucs; Tran Van Bang 2013: Feeding ecology of Annamese silvered langurs; Ulibarri 2013: Socioecology of red-shanked doucs; Le Khac Quyet 2014: Positional behavior of Tonkin snub-nosed monkeys; Clayton 2015: Gut microbiome of red-shanked doucs; Le Hong Thia *et al.* 2015: Feeding ecology of Indochinese silvered langurs) from both Vietnamese and foreign researchers. There is, however, a lack of data on population genetic variability of primates in Vietnam, with only a single study on the critically endangered Delacour's langurs (Ebenau *et al.* 2011). An assessment of the viability and conservation status of primates will be more complete if we also have data on population genetics.

1.6. Research Questions and Hypotheses

I carried out research on three species of colobine monkeys across six sites in Vietnam: four sites for the black-shanked douc (Ta Kou Nature Reserve, Cat Tien National Park, Nui Chua National Park, and Hon Heo Mountain), one site for Indochinese silvered langur (Kien Luong Karst Area where the langur occurs on four isolated hills of Chua Hang, Khoe La, Lo Coc, and Mo So), and one site for Tonkin snub-nosed monkey (Khau Ca Area).

This study addresses three primary research questions on population genetics, dietary profiles, and conservation:

1. **What is the genetic variability of populations of the black-shanked douc (*Pygathrix nigripes*), Indochinese silvered langur (*Trachypithecus germaini*), and Tonkin snub-nosed monkey (*Rhinopithecus avunculus*) in Vietnam? How do factors such as population size and gene flow explain observed patterns of genetic variability?**

   (a) There may be between 3,300 and 4,000 black-shanked doucs in Vietnam (H. Covert and Hoang Minh Duc estimate from 2008) but most of the remaining individuals are found in small, isolated forest fragments (Rawson *et al.* 2008). One of the largest populations of black-shanked doucs in Vietnam is found in Nui Chua National Park with an estimated number of 500-700 individuals (Hoang Minh Duc & Ly Ngoc Sam 2005; Hoang Minh Duc 2007). The population in Cat Tien National Park numbered at 109 individuals more than 10 years ago (Phan Duy Thuc *et al.* 2005) but recent study suggests that there may be more (O'Brien 2014). In Ta Kou Nature Reserve, it was estimated that there were at least 64 black-shanked doucs (Hoang Minh Duc *et al.* 2010a). Little research and/or conservation attention has been given to the unprotected population of black-shanked doucs at the Hon Heo Mountain, hence there is no population estimate at the moment. Overall, based on estimated population size in Vietnam, I expect the black-shanked doucs to retain relatively high genetic variability but limited gene flow between all four populations examined given that they are completely isolated from each other (77 – 232 km between any two sites with areas of human modified landscapes).

   (b) Among 21 isolated karst hills within the Kien Luong Karst Area, the Indochinese silvered langurs are only found on four, with an estimated population size of 286; 131

individuals on Chua Hang hill, 109 on Khoe La hill, 25 on Lo Coc hill, and 21 on Mo So hill (Tran Van Bang *et al.* submitted). In the Indochinese silvered langurs, I expect relatively lower genetic variability as compared to the black-shanked doucs and also limited gene flow between all four populations examined given that they are completed isolated from each other (3 – 9 km between any two hills).

(c) The Tonkin snub-nosed monkey is considered one of the world's most threatened species (Baillie & Butcher 2012) and one of the most endangered primates (Le Khac Quyet *et al.* 2015). At one point, the lack of field reports led the international community to believe that this species might be extinct (Mittermeier & Cheney 1987). Today, it is estimated that there are <250 Tonkin snub-nosed monkeys left in five isolated locations in northeastern Vietnam, with the largest population of 125-130 individuals in the Khau Ca Area (see Le Khac Quyet *et al.* 2015). I expect limited genetic variability given that the original population was small and that a recent doubling of the population from 50–60 to 125–130 individuals (Le Khac Quyet *et al.* 2015) without immigration has been reported. Of all snub-nosed species (*Rhinopithecus* spp.), the Tonkin snub-nosed monkey has the lowest population estimate: *R. roxellana* with approximately 15,000 individuals has the largest population, followed by *R. bieti* with a maximum of 2,000 individuals, *R. brelichi* with 750-800 individuals, and *R. strykeri* with 260-330 individuals in Myanmar and 490-620 individuals in China which include some cross-boundary groups that may range between the two countries (Ma *et al.* 2014; see Yang *et al.* 2012). As the Tonkin snub-nosed monkey has the smallest population size and the most restricted distribution of the snub-nosed monkeys, I expect the lowest genetic variability in the Tonkin snub-nosed monkey as compared to the rest (*R. bieti*, *R. brelichi*, and *R. roxellana*; no population genetic data is available for *R. strykeri*).

2. What are their dietary profiles as revealed by DNA metabarcoding of fecal samples? How do the results compare with field observations? How do habitat flora diversity, habitat differences, anthropogenic disturbances, and seasonality affect dietary diversity?

(a) In order to identify the plants consumed by the monkeys, plant DNA (P6 loop of chloroplast trnL) was obtained from monkey fecal samples through DNA metabarcoding in association with next-generation sequencing. These DNA sequences were then identified through matching against a global genetic database (GenBank). Given that no large plant barcoding and sequence submission projects had been carried out on my study sites and that the P6 loop is small which means the taxonomic resolution at the species level is low, I expect the taxonomic resolution of the sequences to be low (i.e. most sequences may only be identified to family/genus and not to species). I also created local databases by retrieving from GenBank only sequences from plants known to be found in each of my study sites (genus only and species only). I expect the taxonomic resolution of the sequences to be higher (i.e. most sequences may be identified to genus/species) when using the local databases. Overall, I aim to describe the diet of the monkeys at least to the family level. The dietary profiles obtained through DNA metabarcoding was then compared with feeding observations from the field.

(b) The flora diversity within the habitats of the black-shanked doucs in Ta Kou Nature Reserve (Luu Hong Truong 2000, 2006) and Cat Tien National Park (Cat Tien National Park 2012), the Indochinese silvered langurs in Kien Luong Karst Area (see Nguyen Xuan Dang 2009), and the Tonkin snub-nosed monkeys in Khau Ca Area (Vu Anh Tai *et al.* 2013) has been documented. The dietary diversity of the monkeys

gleaned from metabarcoding was compared with the flora diversity within their habitats to examine selectivity.

(c) Fecal samples of the black-shanked doucs were collected from four different sites across Vietnam: (a) evergreen and semi-deciduous sub-mountain forest and broad-leaved lowland forest in Ta Kou Nature Reserve; (b) evergreen, semi-evergreen, mixed tree and bamboo forest, scrub and grassland in Cat Tien National Park; (c) thorny scrub and woodland, dry deciduous forest, sclerophyll evergreen and sub-mountain evergreen forest and tropical savannah woodland in Nui Chua National Park; and (d) tropical dry forest in Hon Heo Mountain. Given the diversity of habitats that the populations inhabit, I expect different dietary profiles of the black-shanked doucs from the four sites.

(d) Fecal samples of the Indochinese silvered langurs, particularly the population from Chua Hang hill, were collected over three years during the wet season. I expect the dietary profiles of the same population to be consistent over the years given that no major anthropogenic disturbance nor climatic events occurred that would alter the flora diversity and composition in Chua Hang hill during my sample collection period (2012 – 2014). On the other hand, Khoe La hill has been under limestone quarrying pressures for cement production since 1998 (International Finance Corporation 2006) and today, more than half of the forested area has been denuded. Diet of the langur population in Khoe La was analyzed and compared with that of Chua Hang population, and I expect the Khoe La population to exhibit less dietary diversity in relation to Chua Hang population.

(e) Fecal samples of the Tonkin snub-nosed monkeys in Khau Ca Area were collected over two months during the transition between wet to dry seasons. Given the marked seasons and seasonal availability of food resources in Khau Ca Area (Le Khac Quyet

2014), I expect to detect a difference in plant taxa recorded in the samples between the two months.

3. How can studies on genetic variability and diet of these populations help with their conservation?

(a) Small, isolated populations rapidly lose genetic diversity through genetic drift, inbreeding, and demographic and environmental stochasticity. Diminished genetic variation reduces the opportunity for adaptive responses to local conditions and may result in reduced reproduction or survival and thereby reduces the viability of the population. Population genetics is one important indicator of population viability of threatened species especially those with small, isolated populations. I aim to provide the first information on mitochondrial genetic variability of three species of colobine primates which is important for assessing their conservation status. This information can then help local conservation agencies and management boards to prioritize targeted conservation actions and strategies in the long term.

(b) When a habitat is being altered, for instance, by logging or quarrying activities, the flora diversity and composition change. The impact of such habitat modifications and the ability of its inhabitants, in this case, the colobine monkeys, to adapt to its changing landscape need to be assessed so as to prioritize mitigation measures and conservation actions. Identifying food species that the monkeys select, especially before, during, and after habitat alteration thus becomes an important step. However, habitat modification usually happens before such an assessment can be made. Alternatively, we may examine diets of two populations whose original habitats were similar (e.g. of the Indochinese silvered langurs inhabiting the various limestone hills in the Kien Luong Karst Area), but one currently undergoing habitat changes (e.g. Khoe La hill) while the other remains relatively undisturbed (e.g. Chua Hang hill).

Here, I expect to detect a difference in dietary profiles of the two populations of Indochinese silvered langurs and I aim to use this information to help with habitat recovery efforts such as identifying food plant species to be planted in the disturbed habitat.

1.7. Research Significance

In this study, I provide the first information on mitochondrial genetic variability of populations for three threatened primate species in Vietnam: black-shanked douc, Indochinese silvered langur, and Tonkin snub-nosed monkey. Different factors influencing genetic variability such as sex-biased dispersal and historical gene flow are examined. Results from this study are expected to contribute not only to a better understanding of population genetics, but also to developing conservation strategies and priorities as it relates to long-term population viability. This is also a first study in which the diets of these three primate species are characterized using a DNA-based approach combining barcoding and next-generation sequencing technologies.

One of the challenges in studying endangered species, especially populations which are also unhabituated and typically shy such as the colobine primates, is that traditional field observational methods are time-consuming and cost-ineffective if population-level data is needed. It is also not feasible to collect certain types of information such as population genetic structure in order to examine its implication for the long-term viability of the species using field observations. Therefore, this study brings value in testing and highlighting the importance of using other methods such as genetic analyses to complement traditional observational methods.

Finally, through this dissertation, I strive to bring to attention the need to apply targeted strategies and recommendations to each conservation issue facing different populations and species of primates. In order to specify and prioritize conservation and

management actions, we need knowledge of extrinsic threats such as pressures from habitat destruction, forest conversion, and hunting, and also intrinsic threats such as restricted gene flow due to isolation of populations. Through the case studies on three species of colobine primates, I identify distinct threats and emphasize the need to provide targeted solutions in order to successfully conserve them.

1.8. Thesis Outline

Chapter I introduces the Asian colobines and the conservation problems that they are facing. The basis of my research aims is illustrated with an overview of population genetics and primate diet and their importance towards conservation. Chapter II describes the study subjects, study sites, and fecal sample collection. Chapter III provides the information on genetic variability in colobine primates and the methods for obtaining and analyzing genetic data. Results of genetic variability of the three study species are evaluated and discussed. Chapter IV describes the emerging field of diet metabarcoding especially in primates and the methods and bioinformatics analyses used. The final dataset of food plant sequences obtained through metabarcoding of fecal samples is presented. Chapters V, VI, and VII detail the results of diet metabarcoding for black-shanked doucs, Indochinese silvered langurs, and Tonkin snub-nosed monkeys respectively, and evaluate their dietary profiles in relation to (1) feeding observations from the field and (2) floristic diversity within their habitats. These chapters also discuss the results in light of population differences, effects of habitat disturbances, and diet transition between seasons. Finally, Chapter VIII summarizes the findings and reviews the conclusions as they relate to our current understanding of population genetics and dietary profiles of Asian colobines. Conservation implications of this research and recommendations for future studies are also presented.

CHAPTER II
STUDY SUBJECTS, SITES, AND SAMPLE COLLECTION

This dissertation on population genetic variability and diet metabarcoding was carried out for three species of colobine primates across six study sites in Vietnam (Fig. 2.1). Four of these sites are for the black-shanked douc (*Pygathrix nigripes*): Ta Kou Nature Reserve, Cat Tien National Park, Nui Chua National Park, and Hon Heo Mountain; one site is for the Indochinese silvered langur (*Trachypithecus germaini*): Kien Luong Karst Area (which includes four isolated hills of Chua Hang, Khoe La, Lo Coc, and Mo So); and one site is for the Tonkin snub-nosed monkey (*Rhinopithecus avunculus*): Khau Ca Area.

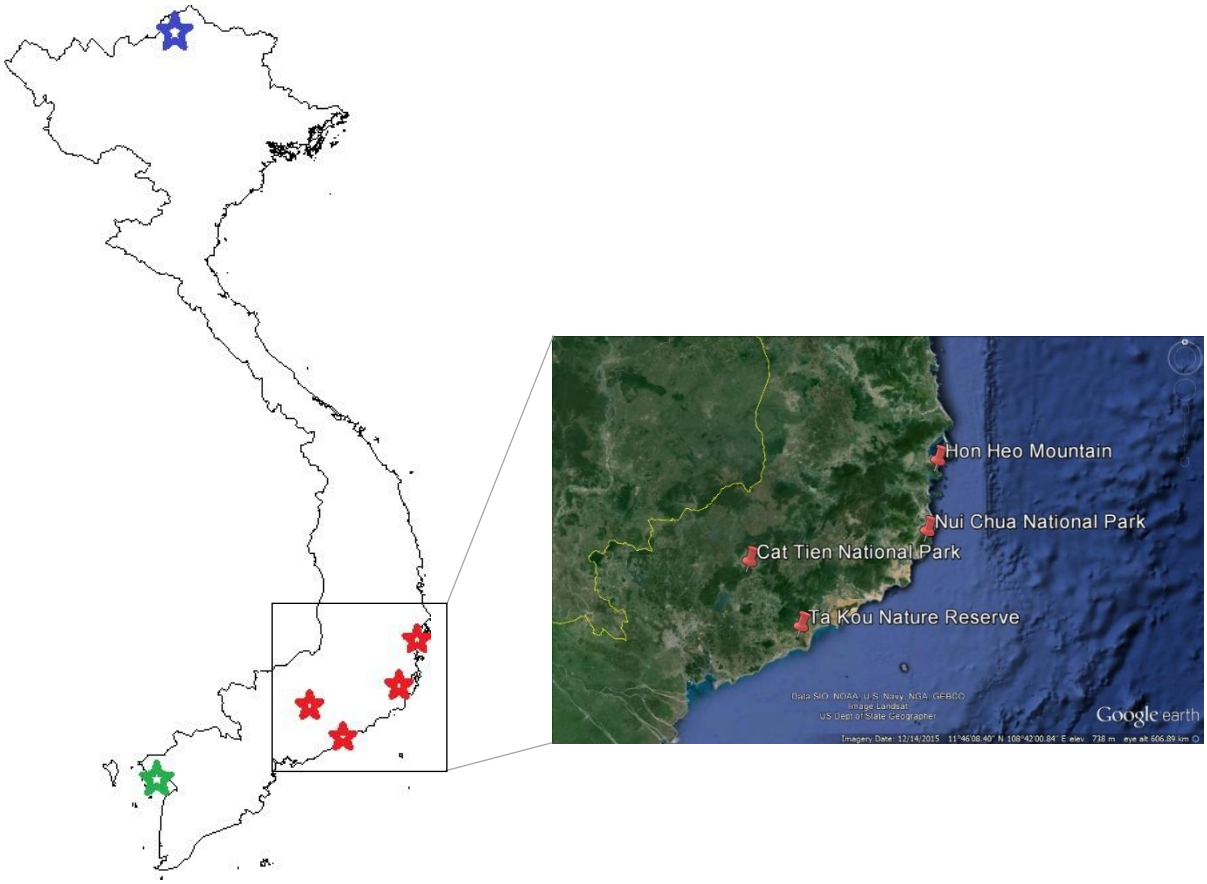


Figure 2.1. Locations of study sites across Vietnam (Image modified from aneki.com). Blue star indicates study site (Khau Ca Area) for the Tonkin snub-nosed monkey, red stars for the black-shanked douc, and green star (Kien Luong Karst Area) for the Indochinese silvered langur.

2.1. The Black-shanked Douc (*Pygathrix nigripes*)

Figure 2.2. Black-shanked doucs in Nui Chua National Park (Photo from Hoang Minh Duc with permission).

The black-shanked douc is one of three species of doucs (*Pygathrix cinerea*, *P. nemaeus*, and *P. nigripes*), distinguished morphologically by having a blue muzzle, spectacled appearance and black shanks (Fig. 2.2). It is endangered with a restricted range in southern Vietnam and northeastern Cambodia (Rawson *et al.* 2008). While a global population estimate is currently not available, it is believed that the largest population of black-shanked doucs is found in Seima Biodiversity Conservation Area (SBCA) in Cambodia, where distance estimates provided a population estimate of approximately 42,000 individuals (95% confidence interval of 27,309-66,460) (Pollard *et al.* 2007).

In Vietnam, it is estimated that there are between 3,300 and 4,000 black-shanked doucs (H. Covert and Hoang Minh Duc estimate from 2008) but most of the remaining individuals are found in small, isolated forest fragments (Rawson *et al.* 2008). The largest population may be in Bu Gia Map National Park in Binh Phuoc Province, followed by Nui Chua National Park in Ninh Thuan Province which was estimated at 500-700 individuals (Hoang Minh Duc & Ly Ngoc Sam 2005; Hoang Minh Duc 2007). A significant population

of 109 individuals was estimated from Cat Tien National Park (Phan Duy Thuc *et al.* 2005), although recent study suggests that there may be more (O'Brien 2014). They are also found in Ta Kou Nature Reserve in Binh Thuan Province, the most southerly extent of the species' distribution (Hoang Minh Duc *et al.* 2008). At least 64 black-shanked doucs were estimated from this protected area (Hoang Minh Duc *et al.* 2010a). They are also found in several unprotected areas such as Hon Heo Mountain in Khanh Hoa Province. In my study of black-shanked doucs, four sites were chosen:

2.1.1. Ta Kou Nature Reserve

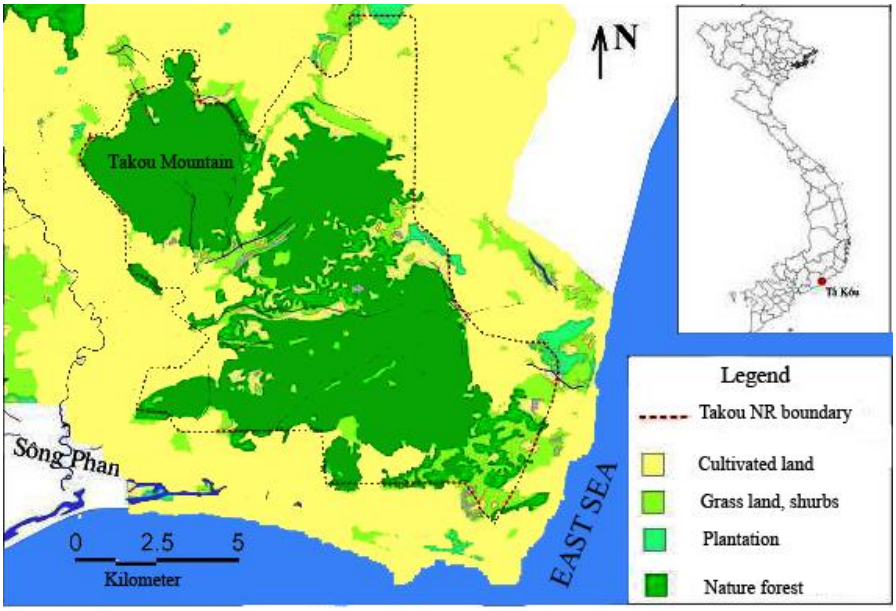


Figure 2.3. Map of Ta Kou Nature Reserve (Image from Tran Van Bang 2013 with permission).

Ta Kou Nature Reserve (Fig. 2.3) in Binh Thuan Province is located in the southern coastal region where it experiences tropical monsoon climate with two distinct seasons based on rainfall. The dry season runs from November to April and the rainy season from May to October. The vegetation consists mainly of three forest types: tropical evergreen seasonal sub-mountain forest, tropical semi-deciduous sub-mountain forest and tropical drought-deciduous broad-leaved lowland forest (Luu Hong Truong 2000, 2001). The strictly protected core zone covers 119 km^2 which consists of 10 km^2 Ta Kou Mountain in the northwestern

part and a 108 km^2 large coastal sandy flat area. Recent studies on ecology and conservation of black-shanked doucs and sympatric Annamese silvered langurs (*T. margarita*) were conducted on Ta Kou Mountain (Hoang Minh Duc *et al.* 2010a; Tran Van Bang 2013).

2.1.2. Cat Tien National Park

Figure 2.4. Map of Cat Tien National Park (Reprinted from www.namcattien.org).

Cat Tien National Park is located in southern Vietnam, in the provinces of Dong Nai, Lam Dong, and Binh Phuoc. This park covers approximately 740 km^2 and is one of the few remaining tracts of lowland evergreen forest in Vietnam. This park receives a high annual rainfall of 2.5 m (Nguyen Xuan Dang *et al.* 2005) and experiences distinct dry (December to April) and wet (May to November) seasons. The vegetation types are diverse, consisting of tropical evergreen forest, semi-evergreen forest, bamboo forest, mixed tree and bamboo forest, scrub, grassland, and wetlands and lakes (Monastyrskii 2000). For this study, only Nam Cat Tien (383 km^2) in the eastern part of southern Vietnam in Dong Nai Province was included (Fig. 2.4). Studies on feeding behavior of black-shanked doucs had been carried out in this park (Phan Duy Thuc *et al.* 2005; O'Brien 2014).

2.1.3. Nui Chua National Park

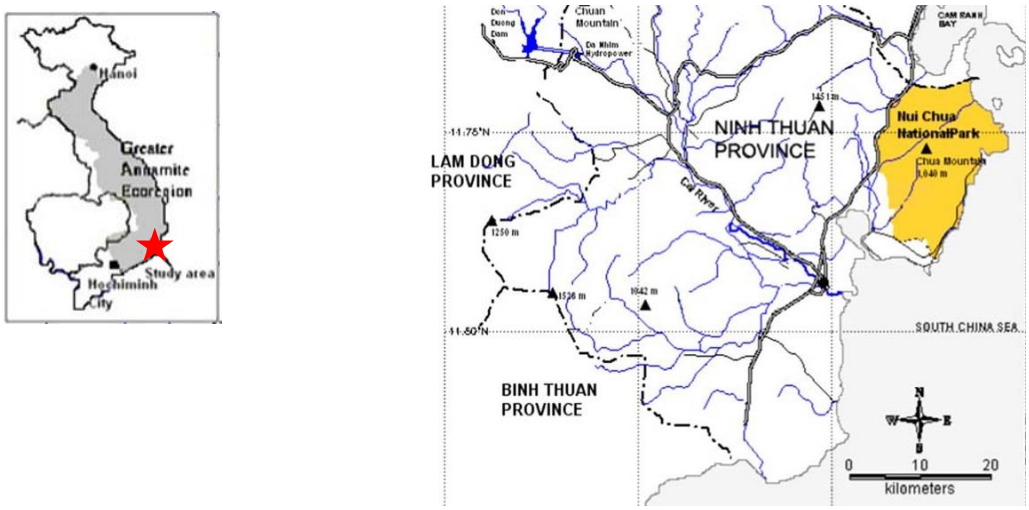


Figure 2.5. Map of Nui Chua National Park (Image from Hoang Minh Duc *et al.* 2009 with permission).

Also located in southern Vietnam, Nui Chua National Park in Ninh Thuan Province covers an area of 29,865 ha, of which the mainland area is 22,513 ha and the sea area is 7,352 ha (Nui Chua National Park 2005). The park is situated within the driest part of Vietnam, receiving an annual average rainfall of only 697 mm (Sub-FIPI 1996). The habitat is characterized by five main vegetation zones: thorny scrubland, dry thorny scrub and woodland, dry deciduous forest and tropical savannah woodland, sclerophyll evergreen forest, and sub-montane evergreen forest.

2.1.4. Hon Heo Mountain

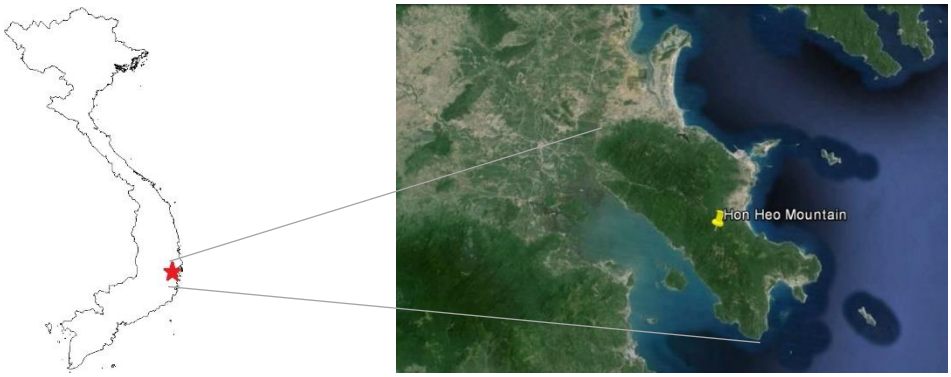


Figure 2.6. Map of Hon Heo Mountain. (Images from aneki.com [left] and Google Earth [right]).

Hon Heo Mountain in Khanh Hoa Province is located in southern Vietnam, covering an area of approximately 15,000 ha with mainly tropical dry forest (Ha Thang Long & Nadler 2007; Fig. 2.6). In 2007, the owner of a tourist resort at the foot of Hon Heo Mountain reported sightings of doucs that did not fit the typical colorations of black-shanked douc (blue face, dark-grey forearms, and black lower legs) nor red-shanked douc (orange face, white forearms, and red lower legs), but were variations of both (such as blue face, white forearms, and red lower legs) (Nadler 2008). This observation prompted an investigation into the genetic affinities of this population among the douc species (*Pygathrix* spp.), and it was concluded that the Hon Heo population is the black-shanked douc (*P. nigripes*) based on mitochondrial d-*loop* sequences (Nadler 2008). Due to a lack of further research on the biodiversity of Hon Heo Mountain, which is an unprotected area, little is known about the habitat and its wildlife. This field site was not originally included in my dissertation so fecal samples of black-shanked doucs were only collected opportunistically.

2.2. The Indochinese Silvered Langur (*Trachypithecus germaini*)

Figure 2.7. Indochinese silvered langur feeding in Kien Luong (Photo: Andie Ang).

The total population of Indochinese silvered langurs is unclear within its geographical range in southern Myanmar, southern Thailand, southern Laos, Cambodia, and the southern tip of Vietnam (see Roos *et al.* 2013) as a result of limited surveys and they are listed as endangered (Hoang Minh Duc *et al.* in prep.). In Vietnam, the Indochinese silvered langurs (Fig. 2.7) are recorded in Kien Luong Karst Area and Phu Quoc National Park in Kien Giang Province, Cape Ca Mau and U Minh Ha National Parks in Ca Mau Province, and in the Seven Mountain Range in An Giang Province. The total population size in Vietnam is estimated to be 362-406 individuals (Tran Van Bang *et al.* submitted), with the largest population of 286 individuals recorded from Kien Luong Karst Area. Continued research efforts by the Southern Institute of Ecology (SIE) of the Vietnam Academy of Science and Technology (VAST) over the past six years have generated the most up-to-date distribution of this species especially in Kien Luong.

2.2.1. Kien Luong Karst Area

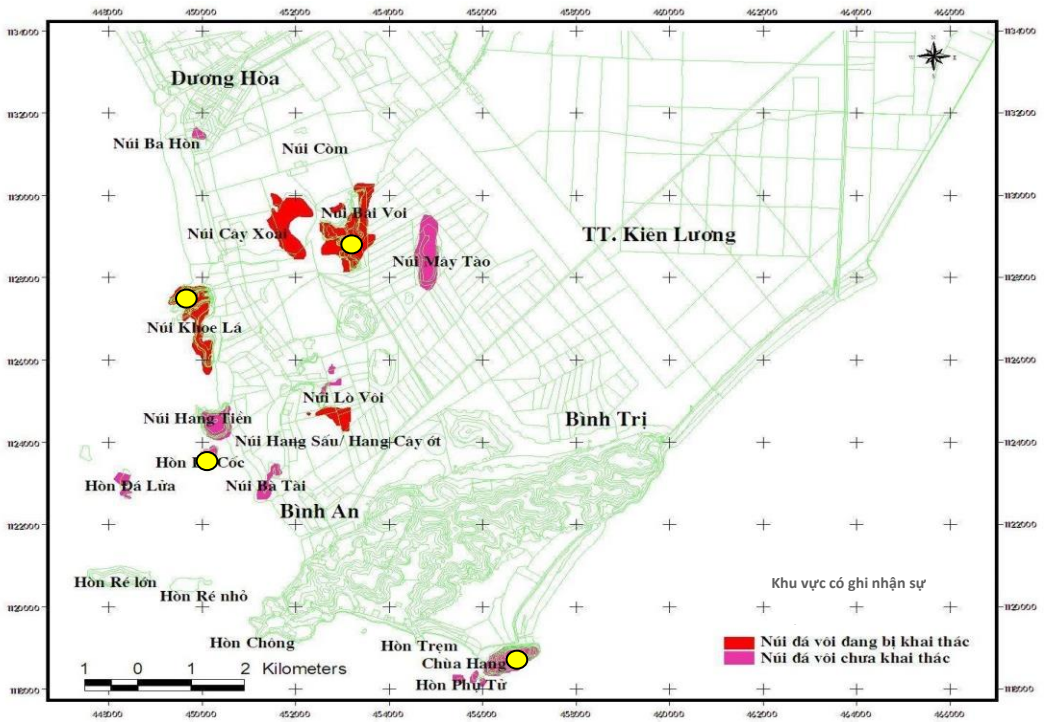


Figure 2.8. Map of Kien Luong Karst Area.
Yellow circles identify location of karst hills where Indochinese silvered langur occurs.
(Image from Hoang Minh Duc *et al.* 2010c with permission).

Kien Luong Karst Area (Fig. 2.8) is located in the delta province Kien Giang, experiencing a tropical monsoon climate with two seasons: rainy from May to November and dry from December to April (Ministry of Culture, Sports and Tourism 2014). It is characterized by four main vegetation types: limestone forests, *Melaleuca* forests and grasslands, mangroves, and coastal shrublands (Nguyen Xuan Dang 2009). The karst area plays an important role in maintaining the viability of the species, holding one of the two largest populations in Vietnam, the other being on Phu Quoc Island, 50 km west of Kien Luong (Nadler *et al.* 2010). Within Phu Quoc National Park, it is estimated that there are 54 Indochinese silvered langurs (Tran Van Bang *et al.* submitted). Collaborative research efforts between SIE and the University of Colorado Boulder are in place so as to monitor the population and conservation status of the species on Phu Quoc Island (e.g. Fiore 2015).

Among the 21 karst hills in Kien Luong, the Indochinese silvered langurs are found on four, with 131 individuals in Chua Hang, 109 in Khoe La, 25 in Lo Coc, and 21 in Mo So (Tran Van Bang *et al.* submitted). These hills are isolated from each other, with Khoe La and Mo So currently being quarried for limestone, forcing the langurs to retreat into smaller limestone pockets with degraded forest cover (Hoang Minh Duc *et al.* 2010c). Conservation initiatives are being developed to assess whether translocation, setting up corridors between karst hills or expansion of protected hills could conserve this endangered species from local extinction. Examining the genetic variability in these small populations will aid in assessing inbreeding risk and their conservation status so as to inform proposed conservation strategies.

2.3. The Tonkin Snub-nosed Monkey (*Rhinopithecus avunculus*)

Endemic to Vietnam, the Tonkin snub-nosed monkey (Fig. 2.9) is critically endangered (Le Xuan Canh *et al.* 2008) and a global priority for primate conservation. Following the first discovery of Tonkin snub-nosed monkey in 1911 and subsequent collections, the lack of field reports led the international community to believe that this

species might be extinct (Mittermeier & Cheney 1987). However, in 1989 a population was discovered in Na Hang-Chiem Hoa region of Tuyen Quang Province (Ratajszczak *et al.* 1992), and in 2002 another population of 50-60 individuals was confirmed in Khau Ca Area, Ha Giang Province (Le Khac Quyet 2002) which was north of the formerly known historical distribution (Ratajszczak *et al.* 1992; Fooden 1996). Currently, the <250 surviving individuals are found in five isolated locations in two provinces of northeastern Vietnam (see Le Khac Quyet *et al.* 2015): 125-130 individuals in Khau Ca Area and 20 individuals in Tung Vai Forest, both in Ha Giang Province; 8-12 individuals in Cham Chu Nature Reserve and 18-26 individuals in Na Hang Nature Reserve (consisting of two isolated populations), both in Tuyen Quang Province.

Figure 2.9. Tonkin snub-nosed monkeys in Khau Ca Area (Photo from Le Khac Quyet with permission).

The Tonkin snub-nosed monkey is not only considered one of the world's 100 most threatened species (Baillie & Butcher 2012) and one of the top 25 most endangered primates (Le Khac Quyet *et al.* 2015), but also the species with the lowest population estimate of all snub-nosed species: *Rhinopithecus roxellana* with approximately 15,000 individuals has the

largest population, followed by *R. bieti* with a maximum of 2,000 individuals, *R. brelichi* with 750-800 individuals, and *R. strykeri* with 260-330 individuals in Myanmar and 490-620 individuals in China which includes some cross-boundary groups that may range between the two countries (Ma *et al.* 2014; see Yang *et al.* 2012). The Tonkin snub-nosed monkey is also one of two species (the other being *R. strykeri*) without any data on intra-population genetic variability.

The largest single population of Tonkin snub-nosed monkey consists of 125-130 individuals in the Khau Ca Area of Ha Giang Province, and infants have been observed in this population for the last 10 years (Le Khac Quyet, personal communication). This population in Khau Ca Area is likely the most important for the survival of the species also because the habitat is better protected, the population is continuously monitored, and conservation education programs are in place (Le Khac Quyet *et al.* 2015). For this reason, Khau Ca Area is an ideal location to collect data on the population genetic variability and dietary profile of the species.

2.3.1. Khau Ca Area

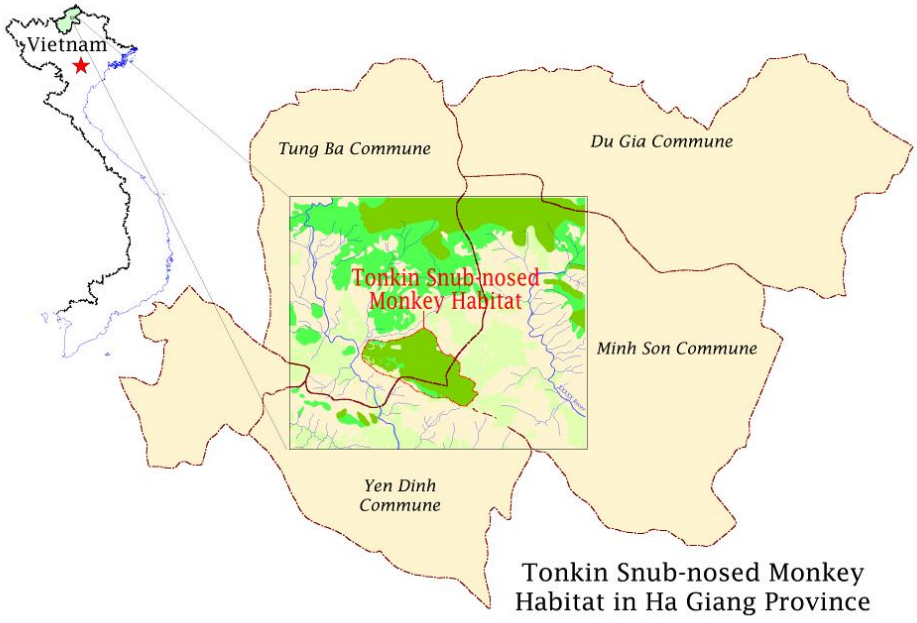


Figure 2.10. Map of Khau Ca Area (Image from Le Khac Quyet *et al.* 2007 with permission).

Khau Ca Area is located in northern Vietnam (Fig. 2.10), ranging in altitude from 600 to 1,400 m asl. With an annual rainfall of about 2.3 m, this sub-tropical region has warm, wet (April–September) and cool, dry (October–March) seasons (Covert *et al.* 2008; Le Khac Quyet 2014). The terrain is characterized by steep and irregular limestone karst, with its 1,000 ha forest dominated by lower montane evergreen limestone forest. While secondary forest, savannah scrub and grassland also occur, higher elevations are dominated by primary and secondary evergreen forest. This site is part of a long-term research focus for the Tonkin snub-nosed monkey, generating important ecological understanding of the species and its habitat (e.g. flora: Vu Anh Tai *et al.* 2013; diet: Le Khac Quyet *et al.* 2007; positional behavior: Le Khac Quyet 2014).

2.4. Fecal Sample Collection

Given that it is extremely risky and difficult to acquire fresh blood or hair samples from unhabituated and rare arboreal primates, fecal samples were collected. A total of 395 fecal samples were collected; 63 from black-shanked doucs (June 2012 and March-May 2014), 78 from Indochinese silvered langurs (July 2012, June 2013 and April 2014), and 254 from Tonkin snub-nosed monkeys (September-October 2014). Existing trails at each field site were walked from dawn till dusk. Whenever the monkeys were observed, they were followed for as long as possible until they rested for the night in order to identify their roosting trees. Whenever defecation was observed, samples were collected immediately after the monkeys left. Fecal samples were also searched for from underneath roosting trees in the mornings after the monkeys left. To minimize the probability of collecting several samples from the same individuals, only samples with a distance of at least 2 m from each other were taken. This method was found to effectively retrieve 96.6% unique samples of *R. brelichi* at any point of collecting (141 individuals of 146 samples after genotyping at eight microsatellite loci) while it cannot avoid sampling same individuals at different times (Yang *et al.* 2012).

Fecal samples were stored using the two-step ethanol-silica method because it has been demonstrated to recover higher amount of DNA from feces compared to traditional methods (Table 2.1) such as storing only in silica or only in 70%-90% ethanol under field conditions (Nsubuga *et al.* 2004).

Table 2.1. Commonly used methods of fecal sample collection for DNA analysis

Collection method	**Storage method**	**Reference**
Dry ziplock bags	-70°C	*Presbytis femoralis*; Ang 2010
Silica beads	4°C	*Brachyteles arachnoides*; Chaves *et al.* 2006
Plastic tubes with 70% ethanol	Room temperature	*Rhinopithecus brelichi*; Yang *et al.* 2012
Plastic tubes with 95% ethanol	Room temperature	*Rhinopithecus bieti*; Liu *et al.* 2007
Two-step ethanol-silica[1]	-80°C	*Colobus angolensis*; McDonald & Hamiliton 2010 *Procolobus rufomitratus*; Mbora & McPeek 2010 Miyamoto *et al.* 2013
RNALater[2]	Room temperature	*Colobus angolensis*; McDonald & Hamiliton 2010 *Nasalis larvatus*; Munshi-South & Bernard 2011

[1]Fecal sample are placed into a tube containing 30ml of 100% ethanol. After 24-36 hours, the ethanol is carefully poured off with the tube loosely capped, and the remaining solid material is transferred into a new-labeled tube containing silica for drying and storage. This two-step method (as compared to only storing in ethanol or only in silica) aims to further dessicate samples in order to stop hydrolytic degradation of DNA. Samples are then stored at -80°C in laboratory.
[2]RNALater (Ambion, Austin, TX) is a buffer containing ammonium sulphate salt which preserves the integrity of RNA in tissue by precipitating endogenous RNases and other soluble proteins. Samples store for one day at 37°C, for 7 days at 15-25°C, for 4 weeks at 2-8°C, and for years at -20°C or -80°C.

Figure 2.11a. Fecal sample of Indochinese silvered langur in the sandy coastal area of Kien Luong Karst Area (Photo: Andie Ang).

Figure 2.11b. Fecal sample of Tonkin snub-nosed monkey in a limestone crevice in Khau Ca Area (Photo: Andie Ang).

Collections at different field sites presented different challenges: in coastal Kien Luong, windy condition meant that many samples were covered with sand (Fig. 2.11a), which necessitated careful removal before collection; in the limestone forests of Khau Ca, samples occasionally fell into crevices (Fig. 2.11b), which demanded careful maneuvering on top of sharp limestone karsts in order to retrieve.

CHAPTER III
GENETIC VARIABILITY OF COLOBINE PRIMATES

3.1. Introduction

Genetic variability is regarded as an important factor for the long-term survival of a species (Dietz *et al.* 2000; Di Fiore 2003; Höeglund 2009). Small populations rapidly lose genetic variability through genetic drift and inbreeding (Lande & Barrowclough 1987). Diminished genetic variability within populations, or loss of distinct populations, reduces the opportunity for adaptive responses to geographically varying local conditions (Sherwin & Moritz 2000), and may result in reduced reproduction or survival and thereby reduces the viability of the population (Nei *et al.* 1975; Madsen *et al.* 1996). Understanding genetic variability can thus help assess a species' viability and contribute to its conservation.

Molecular genetic analyses of primates had been limited by the availability of blood or tissue samples for DNA extraction (see Surridge *et al.* 2002) until Boom *et al.* (1990) presented the first study that was successful in isolating DNA from shed epithelial cells on the surface of feces. Since then, genetic studies using DNA from fecal samples have been carried out in many primate species in the wild. The first step in any analysis of genetic variation involves characterizing individuals using molecular markers (see Di Fiore 2003). Different genetic markers and analytical techniques can be used to investigate genetic variability and structure. The hypervariable region I (HV-I) of the displacement loop (d-*loop*) of the mitochondrial DNA is often used to evaluate the level of genetic diversity in primate populations (see Tables 3.1 and 3.7) because (1) HV-I is the most rapidly evolving part of the mitochondrial genome (Lopez *et al.* 1997); (2) the mutation rate of HV-I is relatively stable in primates (Zhang & Hewitt 2003); and (3) HV-I is a widely used marker in population studies so a comparative database is available (see Pan *et al.* 2009).

Table 3.1. Studies on d-*loop* genetic variability of primates in the wild (non-exhaustive; except colobines)

Taxa		**Sample Location**	**No. of individuals**	**Reference**
STREPSIRRHINI				
	Gray mouse lemur (*Microcebus murinus*)	Madagascar	85	Wimmer *et al.* 2002
			205	Fredsted *et al.* 2004
	Golden brown mouse lemur (*M. ravelobensis*)	Madagascar	114	Guschanski *et al.* 2007
	Northern rufous mouse lemur (*M. tavaratra*)	Madagascar	72	Pais 2011
	Coquerel's sifaka (*Propithecus coquereli*)	Madagascar	82	Bailey *et al.* 2016
	Perrier's sifaka (*P. perrieri*)	Madagascar	51	Bailey *et al.* 2016
	Tattersall's sifaka (*P. tattersalli*)	Madagascar	76	Bailey *et al.* 2016
	Black-and-white ruffed lemur (*Varecia variegata*)	Madagascar	209	Baden *et al.* 2014
PLATYRRHINI				
	Black-and-gold howler (*Alouatta caraya*)	Argentina, Paraguay	73	Ascunce *et al.* 2007
	Mexican howler (*A. palliata mexicana*)	Mexico	45	Dunn *et al.* 2014
	Azara's owl monkey (*Aotus azarai azarai*)	Argentina	118	Babb *et al.* 2011
	Brown spider monkey (*Ateles hybridus*)	Colombia	41	Link *et al.* 2015
	Northern muriqui (*B. hypoxanthus*)	Brazil	152	Chaves *et al.* 2011
	Common marmoset (*Callithrix jacchus*)	Brazil	77	Faulkes *et al.* 2003
	Common woolly monkey (*Lagothrix lagotricha*)	Ecuador	61	Di Fiore 2009
	Moustached tamarin (*Saguinus mystax*)	Peru	69	Huck *et al.* 2007
	Black-handed tamarin (*S. niger*)	Brazil	22	Vallinoto *et al.* 2006
CATARRHINI				
	Western lowland gorilla (*Gorilla gorilla gorilla*)	Across its range in nine countries	83	Clifford *et al.* 2004
	Long-tailed macaque (*Macaca fascicularis*)	Mauritius	82	Kawamoto *et al.* 2008
	Rhesus macaque (*M. mulatta*)	China	231	Xu *et al.* 2010
	Bonobo (*Pan paniscus*)	DR Congo	157	Eriksson *et al.* 2004
	Eastern African chimpanzee (*P. troglodytes schweinfurthi*)	DR Congo, Uganda	281	Goldberg 1998
	Eritrean hamadryas baboon (*Papio hamadryas hamadryas*)	Eritrea	74	Hapke *et al.* 2001
	Bornean orangutans (*Pongo pygmaeus*)	Malaysian Borneo	78	Jalil *et al.* 2008

The golden brown mouse lemur (*Microcebus ravelobensis*) is endangered and endemic to northwestern Madagascar. Populations are separated by two river systems, and further divided into groups within continuous forests and forest fragments. It is crucial to examine how geographic isolation and habitat discontinuity influence population genetic diversity. By examining mitochondrial d-*loop* variability within and among populations of golden brown mouse lemurs in six forest fragments and three continuous forests, Guschanski *et al.* (2007) found a lower genetic variability (1-3 haplotypes/population) in isolated forest fragments than in continuous forests (5-6 haplotypes/population). Moreover, the smaller the forest fragments, the lower the level of genetic diversity (an influence of population density, sex ratio, or sample size could not be detected). These results were also interesting in that the effects of geographic isolation and deforestation on population viability could be detected after a short period of time, as extensive land usage and loss of forest cover probably began only a few hundred years ago (Guschanski *et al.* 2007). Finally, the two river systems were considerable dispersal barriers such that populations were sufficiently differentiated from each other: *M. ravelobensis* might consist of three evolutionary significant units, possibly cryptic species, which warranted urgent conservation efforts (Guschanski *et al.* 2007).

The northern muriqui (*Brachyteles hypoxanthus*) is critically endangered and a flagship species of the Brazilian Atlantic Forest, but like many other Neotropical primates, little is known about its population history and genetic structure. By analyzing the d-*loop* of 152 muriquis from eight of the 12 known wild populations, several interesting conclusions were presented (Chaves *et al.* 2011). First, genetic variability was higher in populations living in larger areas (>2000 ha). Second, genetic variability within each population was remarkably low (haplotype diversity of 0-0.846 and nucleotide diversity of 0-0.013). Third, almost all haplotypes (19 out of 23) were exclusively found in only one of the eight populations. Moreover, four populations harbored 22 of 23 haplotypes, most of which were population-

exclusive and therefore represented patchy repositories of the species' genetic diversity. Hence, Chaves *et al.* (2011) recommended that these four populations be considered as discrete units for conservation.

Across Vietnam, scientific research has provided important information for understanding primate ecology, behavior and conservation (e.g. Hoang Minh Duc *et al.* 2010b: Population status of yellow-cheeked crested gibbon; Workman & Schmitt 2012: Positional behavior of Delacour's langurs; Tran Van Bang 2013: Feeding ecology of Annamese silvered langur; Ulibarri 2013: Socioecology of red-shanked doucs; O'Brien 2014: Ecology and conservation of black-shanked doucs), but there is a lack of data on genetic variability. Populations of primates living in isolated forest fragments have lower chances of gene flow and higher risks of inbreeding. Knowledge of genetic variability to assess gene flow and extinction risks is thus equally crucial to evaluating current conservation management and to predicting future population viability.

However, genetic studies involving colobine primates in Vietnam have previously, and remain, focused on phylogenetic relationships and taxonomic resolutions (e.g. Jablonski & Peng 1993; Zhang & Ryder 1998; Li *et al.* 2004; Roos *et al.* 2007; Liedigk *et al.* 2012; Wang *et al.* 2012), illegal primate trade (e.g. Liu *et al.* 2008), and hybridization genetics (e.g. Schempp *et al.* 2008). There is a lack of population genetics of the colobines in Vietnam, with only a single study on the critically endangered Delacour's langur (*Trachypithecus delacouri*; Ebenau *et al.* 2011). Eighty-five fecal samples of Delacour's langurs from Van Long Nature Reserve (VLNR) and neighboring areas were analyzed, yielding nine haplotypes (Ebenau *et al.* 2011). With an overall haplotype diversity of 0.576, nucleotide diversity of 0.009, and mean number of pairwise nucleotide difference between sequences of 3.366, genetic variability of the population of Delacour's langurs is very low. This first information on the genetic structure and variability of the critically endangered Delacour's langurs was

significant in supporting conservation plans to reintroduce individuals from the Endangered Primate Rescue Center (EPRC) into the VLNR and surrounding areas. The genetic variability of the subpopulations in the eastern portion of the species' distribution (including VLNR) was comparatively similar to that in the EPRC (Ebenau *et al.* 2011) such that reintroduction plans to genetically mix these populations would not result in deleterious effects of outbreeding between genetic dissimilar/distinct individuals. On the contrary, such population management strategy would likely be successful in increasing the genetic variability of the populations and ensuring the long-term persistence of the species.

The main research aims of this chapter are to examine the HV-I genetic variability of three colobine primates in Vietnam: the black-shanked douc, Indochinese silvered langur, and Tonkin snub-nosed monkey and to assess interpopulation gene flow. Additionally, full mitochondrial genomes from five fecal samples of Tonkin snub-nosed monkeys are also examined. Based on estimated population size in Vietnam, I expect the black-shanked doucs to retain relatively high genetic variability (Hypothesis 1a) but limited gene flow between all four populations examined (Hypothesis 1b) given that they are completely isolated from each other. In the Indochinese silvered langurs, I expect relatively lower genetic variability (Hypothesis 2a) and also limited gene flow between all four populations examined (Hypothesis 2b) given that they are completed isolated from each other. In the Tonkin snub-nosed monkeys, I expect limited genetic variability (Hypothesis 3a) given that the original population was small and that a recent doubling of the population from 50–60 to 125–130 individuals (Le Khac Quyet *et al.* 2015) without immigration has been reported. As the Tonkin snub-nosed monkey has the lowest population estimate and the most restricted distribution of all snub-nosed monkeys (*Rhinopithecus* spp.), I also expect this species to have the lowest genetic variability as compared to the rest (Hypothesis 3b) (*R. bieti*, *R. brelichi*, and *R. roxellana*; no population genetic data is available for *R. strykeri*).

3.2. DNA Extraction

Genomic DNA was extracted from feces (see **Chapter 2.4. on Fecal Sample Collection**) using both Qiagen stool kit and standard phenol-chloroform extraction (Kutty *et al.* 2007). For each sample, a piece of ca. 50mg feces was transferred into a tube containing 20µl proteinase K and 500µl CTAB (cetyltrimethylammonium bromide) lysis buffer for digestion and incubation in a heating block at 55 °C for 2-23 hours, followed by adding 500µl phenol-chloroform. The tube was centrifuged for 10 mins at 13,200 rpm to sediment insoluble matter. The supernatant was transferred to a new tube and extracted again with 500µl phenol-chloroform, centrifuging at the same conditions. The supernatant was then transferred to a new tube and DNA was precipitated with 700µl absolute ethanol, stored at -20°C overnight. After centrifuging at 13,200 rpm for 30 mins, the DNA pellet was washed twice with 70% ethanol and dried. Dried DNA was then dissolved in varying amounts of nuclease-free water and stored at -20°C at the molecular facilities at the National University of Singapore. A total of 395 fecal samples were collected (63 from black-shanked doucs, 78 from Indochinese silvered langurs, and 254 from Tonkin snub-nosed monkeys), and I extracted 318 samples (39, 59, and 220 respectively) with the remaining 77 samples not yet extracted due to time constraints.

3.3. DNA Amplification and Sequencing

3.3.1. HV-I/D-loop

Due to a lack of population genetic studies for black-shanked douc (BSD), Indochinese silvered langur (ISL), and Tonkin snub-nosed monkey (TSNM), primers specific to each of the taxa at the HV-I region were not available in published literature. There are, however, HV-I sequences available for all three target species from GenBank (National Center for Biotechnology Information): BSD (HM032730.1, Que *et al.* 2010), ISL (NC_019580.1, Wang *et al.* 2012), and TSNM (JF293093.1, Roos *et al.* 2011). Therefore, I

designed forward and reverse primers that were sufficiently specific to their HV-I region (Table 3.2). I first compared published complete mitochondrial sequences for *Rhinopithecus*, *Pygathrix*, and *Trachypithecus* in GenBank using Sequencher 4.6 (Gene Codes Corporation) and searched for highly conserved regions for primer binding sites. The designed oligonucleotide sequences were tested for functionality and checked against formation of hairpin, self-dimer or hetero-dimer using OligoAnalyzer 3.1 (Integrated DNA Technologies).

Table 3.2. Designed primers for mitochondrial HV-I/d-*loop* for Sanger sequencing

Species	Primer name	Primer sequences (5'–3')	Expected gene size (bp)	Annealing temp. (°C)
Black-shanked douc (*Pygathrix nigripes*)	Avunculus_F1 Trac_primer_R1	GGCAACTCAGAAAGAAAGCAC TGGTTAATAGGGTGATAGACCC	622	54-56
	Trac_primer_F2 Avunculus_R2	ACATCTGGTTCTTACCTCAGGG TAGAAAGGCCGGGACCAAACCT	654-655	54-58
Indochinese silvered langur (*Trachypithecus germaini*)	Trac_primer_F1 Trac_primer_R1	GGCAACTCAGAAAGAAAGTAC TGGTTAATAGGGTGATAGACCC	616	52-54
	Trac_primer_F2 Avunculus_R2	ACATCTGGTTCTTACCTCAGGG TAGAAAGGCCGGGACCAAACCT	658	54-58
Tonkin snub-nosed monkey (*Rhinopithecus avunculus*)	Avunculus_F1 Trac_primer_R1	GGCAACTCAGAAAGAAAGCAC TGGTTAATAGGGTGATAGACCC	622	54-56

HV-I region and entire d-*loop* of mtDNA were sequenced using designed primers (Table 3.2). For each sample, a direct polymerase chain reaction (PCR) amplification was carried out in a total volume of 25µl consisting of 2.5µl buffer (10X 15mM $MgCl_2$), 1.0µl dNTPs (10mM), 1.0µl BSA (1mg/ml), 0.25µl BioReady rTaq DNA polymerase (5 units/µl) (Bulldog Bio. Inc.), 0.9µl of each primer (10µM), 0.5-1.0µl template DNA. Nuclease-free water was added accordingly. Amplification conditions were as follows: denaturation at 95°C: 5 mins, 40 cycles of 1 min at 94°C, 1 min annealing at 52°C to 58°C, 1 min at 72°C, final extension: 5 mins at 72°C. The resulting amplification products were electrophoresed on 1% agarose gels, and visualized by GelRed (Biotium) staining. A negative PCR control was processed along with each set of PCR reactions. A successful PCR reaction was defined as one producing specific product in the expected size range. For Sanger sequencing, amplified products were purified separately using Bioline SureClean (Randolph) and sequenced in both

directions using BigDye ver. 3.1 (Applied Biosystems). After purification with CleanSEQ® kit (Agencourt Bioscience Corporation), direct sequencing was carried out in an ABI PRISM® 3100 Genetic Analyzer (Perkin Elmer Applied Biosystems). Sequencing was also carried out using the NGS barcoding strategy outlined in Meier *et al.* (2016), i.e. with primers (F: 5'-TGGCATTCTATTTAAACTAC-3'; R: 5'-GYCATWTATAGCTACCCCCAC-3') uniquely tagged with seven nucleotides at the 5' end of each primer. NGS sequencing was carried out on Illumina MiSeq (Nano kit 2x300PE) after preparing two TruSeq libraries (June and August 2015) for amplicons, each included all three species.

3.3.2. Mitochondrial Genomes and Nuclear DNA of Tonkin Snub-nosed Monkey

Five fecal samples of TSNMs collected on different dates from different GPS locations within Khau Ca were prepared and sent for direct shotgun sequencing in one lane of Illumina HiSeq 4000 (150PE) following the pipeline in Srivathsan *et al.* (2015, 2016) in order to obtain full mitochondrial genomes. Extracted DNA was size selected using varying amounts of poly-ethylene glycol (PEG-8000) and Sera-Mag SpeedBeads (GE Healthcare Life Sciences) to obtain 200- to 400-bp fragments (Rohland & Reich 2012; Faircloth & Glenn 2014). Libraries were constructed using TruSeq Nano Library preparation kit (Illumina).

I also tested whether nuclear markers developed for *Rhinopithecus brelichi* (Kolleck *et al.* 2013) could be used for *R. avunculus*. I tried eight pairs of primers for microsatellite loci (D1S533, D2S1326, D6S264, D6S501, D7S2204, D8S505, D10S1432, D17S1290; see Kolleck *et al.* 2014 for loci details) on seven samples. PCR amplifications were carried out in 25µl consisting of 2.5µl buffer (10X 15mM $MgCl_2$), 1.0µl dNTPs (10mM), 1.0µl BSA (1mg/ml), 0.25µl BioReady rTaq DNA polymerase (5 units/µl) (Bulldog Bio.), 0.9µl of each primer (10µM), 1.0µl template DNA, and 17.45µl of nuclease-free water. Amplification conditions followed Kolleck *et al.* (2014) with annealing temperature at 50°C-58°C. However, amplifications failed with no visible products on the gel. I next tried a longer

amplification time, identical to that for HV-I/d-*loop* (see above **3.3.1. HV-I/D-*loop***) but at annealing temperature of 50°C-58°C. However, final sequences showed no tandem repeats and did not match to anything in GenBank. Finally, I attempted a different Taq polymerase. PCR amplifications were carried out in 25µl consisting of 2.5µl buffer (10X Mg^{2+} free), 2.0µl dNTPs (10mM), 1.0µl $MgCl_2$ (25mM), 0.15µl ex-Taq DNA polymerase (5 units/µl) (Takara), 1.2µl of each primer (10µM), 1.0µl template DNA, and 15.95µl of nuclease-free water. Final sequences showed no tandem repeats and did not match to anything in GenBank.

3.4. Data Analysis

3.4.1. HV-I/D-loop

A total of 90 samples were sequenced using Sanger platform and sequences were edited in Sequencher 4.6. For NGS, a total of 129 samples were sent in June 2015 and 99 samples in August 2015. Samples were considered successfully sequenced if ≥50 reads were recovered, the dominant read was ≥five times as common as the next common read, and the dominant read had a coverage of ≥10. After applying the bioinformatics filtering steps, a total of 114 samples (88.4%) from the first run and 87 samples (87.9%) from the second run were retained for further analysis. A total of 111,435 read counts were obtained from the 114 samples (i.e. 977.5 reads per sample, highest 4,039 reads and lowest 80 reads) and 167,002 read counts were obtained from the 87 samples (i.e. 1,919.6 reads per sample, highest 8,952 reads and lowest 72 reads). In sum, both Sanger and NGS platforms generated 290 HV-I/d-*loop* sequences (Table 3.3). Finally, I aligned all sequences using multiple alignment fast Fourier transform (MAFFT) online version 7 (Katoh & Kuma 2002).

Table 3.3. Total number of HV-I/d-*loop* sequences

Species	Population/Site	# samples | Sanger	bp	# samples | NGS	Bp	**Total # sequences**
Black-shanked douc	Cat Tien	9	574-1,092	1	467	**10**
	Ta Kou	17	580-1,093	4	467	**21**
	Nui Chua	-	-	3	467	**3**
	Hon Heo	3	581-1,093	-	-	**3**
Indochinese silvered langur	Chua Hang	28	1,091	3	461	**31**

	Khoe La	13	1,091	3	461	**16**
	Lo Coc	3	1,091	-	-	**3**
	Mo So	2	1,091	-	-	**2**
Tonkin snub-nosed monkey	Khau Ca	14	483-650	187	467	**201**
Total # of sequences		**89**		**201**		**290**

Genetic variability within populations of a species was estimated by using haplotype diversity (h), nucleotide diversity (π) (Nei 1987), number of haplotypes (unique sequences), number of variable sites (V), and the average number of pairwise nucleotide differences (Π) (Wakeley 1997) in DnaSP 5.10.1 (Librado & Rozas 2009). Haplotype diversity within a population incorporates both the frequency of each haplotype within the group and the estimated distance among each of its respective pair of haplotypes. Nucleotide diversity is based on the average number of nucleotide differences or substitutions per site within and among groups of DNA sequences (Rosenblum *et al.* 1997). All heteroplasmy detected (genetic code Y, M, etc.) were replaced by the ambiguity code N. Haplotype network analysis was carried out using NETWORK 4.6.1.3 (www.fluxus-engineering.com/sharenet.htm) with median-joining algorithm (Bandelt *et al.* 1999) to illustrate haplotype relationships and examine the association of haplotype groups with geography.

3.4.2. Mitochondrial Genomes and Nuclear DNA of Tonkin Snub-nosed Monkey

Metagenomic shotgun data was quality trimmed to remove low-quality bases (<Q20) and adapter contaminations using Trimmomatic version 0.33 (Bogler *et al.* 2014). Trimmed sequences were mapped onto the reference mitochondrial genome of *R. avunculus* (NC_015485.1, Yu *et al.* 2011) obtained from GenBank using the Burrows-Wheeler (bwa *mem*) alignment tool under default settings (Li & Durbin 2009). Mapped data were filtered to retain sequences with mapping quality of 20 and maximum 5bp mismatch using samtools and NGSUtils (Breese & Liu 2013). FreeBayes (Garrison & Marth 2012) was used for single nucleotide polymorphism (SNP) calling under ploidy=1, minimum coverage of five,

coverage for alternate allele of five and variant quality score of ≥30 to identify variant sites (Srivathsan *et al.* 2016).

Lastly, primate nuclear DNA content in the fecal metagenomes was estimated by mapping metagenomic reads onto assembled scaffolds of a *R. roxellana* genome (GCF_000769185.1, Zhou *et al.* 2014) using Bowtie2 (end-to-end alignment, sensitive settings, Langmead & Salzberg 2012). An initial estimate was based on the number of reads mapped. A second estimate was done after excluding reads having >5bp mismatch with the reference. This threshold was determined by measuring the maximum divergence between *R. avunculus* and *R. roxellana* for 66 available nuclear genome markers in GenBank.

3.5. Genetic Variability of the Black-shanked Douc

A total of 37 HV-I and complete mitochondrial d-*loop* sequences (467-1,093 bp; overlapping region of 461 bp) for black-shanked doucs were obtained. With a total of 52 polymorphic sites among 14 haplotypes, a haplotype diversity of 0.896 ± 0.033, a nucleotide diversity of 0.032 ± 0.003, and an average number of nucleotide differences between sequences of 14.565 (Table 3.4), the genetic variability of black-shanked doucs from Cat Tien and Nui Chua national parks, Ta Kou Nature Reserve, and Hon Heo Mountain was high. For Nui Chua National Park and Hon Heo Mountain, only three fecal samples were collected from each site which precludes further site-specific analyses.

Table 3.4. Genetic variability (HV-I/d-*loop*) of black-shanked doucs

Study sites	**N**	**V**	**H**	***h***	**π**	**Π**
Ta Kou	21	17	4	0.705±0.070	0.014±0.003	6.562
Cat Tien	10	30	7	0.933±0.062	0.026±0.004	12.000
Nui Chua	3	0	1	0	0	0
Hon Heo	3	1	2	0.667±0.314	0.00115±0.00054	0.667
Combined	**37**	**52**	**14**	**0.896±0.033**	**0.032±0.003**	**14.565**

N: Number of individuals/samples; V: number of polymorphic sites; H: number of haplotypes; *h*: haplotype diversity; π: nucleotide diversity; Π: average number of nucleotide differences.

Haplotype H3 had the highest frequency (27%) and was twice as frequent as H6, the second most frequent. Both H3 and H6 were exclusive to Ta Kou Nature Reserve. This distribution likely reflected the joint effects of a sampling bias because Ta Kou encompassed 57% of the total black-shanked douc samples and the lack of recent gene flow between all the populations; there were no shared haplotypes between the four sites. Ta Kou and Nui Chua populations were the most distant from each other with a maximum of 46 mutational steps between H14 and H3/H5. Population in Hon Heo Mountain represented a genetic connection between Nui Chua and the other two sites, Ta Kou and Cat Tien (Fig. 3.1).

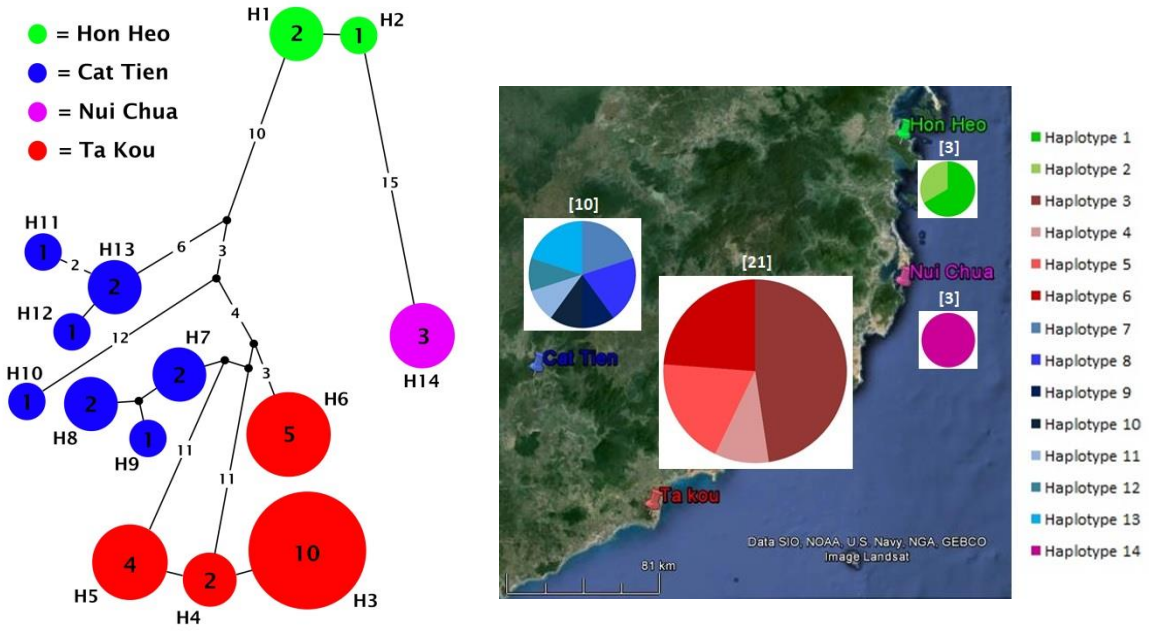


Figure 3.1. Haplotype network and haplotype distribution of black-shanked doucs. Median-joining network depicting haplotype relationships is on the left. Circle sizes are proportional to haplotype frequency (the smallest circle represents 1 sample, and the largest circle represents 10 samples). Each node (small black circles) represents missing intermediate haplotype. The number on each vector line accounts for the number of mutational steps (vector with no number represents one mutational step between the two haplotypes). The map on the right shows the haplotype frequencies (pie charts) within each site/population. Each color represents one of the 14 haplotypes. The chart area is scaled to the sample size (also in brackets).

While a global population estimate is currently not available, the black-shanked douc is recognized as an endangered species with a restricted range in southern Vietnam and

northeastern Cambodia (Rawson *et al.* 2008). In Vietnam, it is estimated that there are between 3,300 and 4,000 black-shanked doucs (H. Covert and Hoang Minh Duc estimate from 2008) but most of the remaining individuals are found in small, isolated forest fragments (Rawson *et al.* 2008). Among the four populations examined in this dissertation, there were population estimates from Ta Kou Nature Reserve (at least 64 individuals: Hoang Minh Duc *et al.* 2010a), Cat Tien National Park (more than 109 individuals: Phan Duy Thuc *et al.* 2005; O'Brien 2014), and Nui Chua National Park (500-700 individuals: Hoang Minh Duc & Ly Ngoc Sam 2005; Hoang Minh Duc 2007); no estimate is currently available for the unprotected Hon Heo Mountain.

When four populations were examined together, the HV-I variability of the black-shanked doucs was high (Hypothesis 1a); it exhibited the highest genetic variability as compared to the Indochinese silvered langur (see below **3.6 Genetic Variability of the Indochinese Silvered Langur**) and Tonkin snub-nosed monkey (see below **3.7 Genetic Variability of the Tonkin Snub-nosed Monkey**). The HV-I variability of the black-shanked douc was also among the highest of colobine primates examined (see Table 3.7). However, there were no shared haplotypes between the four populations (Hypothesis 1b), indicating that recent gene flow between them has been restricted.

The high genetic variability but limited gene flow of the black-shanked douc is similar to another endangered Asian colobine, i.e. China's golden snub-nosed monkey (*Rhinopithecus roxellana*) which has a total population size of approximately 15,000 (see Liedigk *et al.* 2012). The golden snub-nosed monkey is restricted to three isolated areas in the provinces of Sichuan/Gansu, Shaanxi, and Hubei (Li *et al.* 2002). Using d-*loop* sequences, Li *et al.* (2007) identified 12 haplotypes among 60 golden snub-nosed monkeys, indicating that this species had a relatively high genetic variability. However, all populations were distinctly differentiated from each other (e.g. no shared haplotypes) indicating that each of the three

populations should be regarded as management units (MU; see Moritz 1994). Studies based on morphological characters of the populations in the three regions also described some significant differences (e.g. Wang *et al.* 1998). Thus, it was recommended that these three populations should be regarded as separate MUs, with an emphasis on the protection of their habitats (Li *et al.* 2007). Further genetic analyses coupled with phylogeographic patterns should include more sites such as Bu Gia Map National Park in Binh Phuoc Province, which may be home to the largest population of black-shanked doucs in Vietnam, and also Seima Biodiversity Conservation Area (SBCA) in Cambodia, which is believed to be home to the largest population of this species (Pollard *et al.* 2007). Additionally, management units in the black-shanked doucs may need to be identified, especially under the condition of further shrinking distribution and increasing isolation.

3.6. Genetic Variability of the Indochinese Silvered Langur

Of the 52 fecal samples collected from the Indochinese silvered langurs from Kien Luong Karst Area, I generated complete d-*loop* sequences for 44 samples and HV-I sequences for eight samples (461-1,091 bp; overlapping region of 455 bp). With a total of 42 polymorphic sites among six haplotypes, a haplotype diversity of 0.787 ± 0.026, a nucleotide diversity of 0.028 ± 0.003, and an average number of nucleotide differences between sequences of 12.587, the genetic variability of the population of Indochinese silvered langurs on the four limestone hills of Kien Luong was relatively high (Hypothesis 2a). However, each of the four subpopulations exhibited low genetic variability (Hypothesis 2b) when considered separately (Table 3.5).

Table 3.5. Genetic variability (HV-I/d-*loop*) of Indochinese silvered langurs

Study sites	**N**	**V**	**H**	***H***	**π**	**Π**
Chua Hang	31	12	3	0.563±0.051	0.009±0.001	4.142
Khoe La	16	35	3	0.608±0.090	0.031±0.005	14.075
Lo Coc	3	39	2	0.667±0.314	0.024±0.011	26.000
Mo So	2	0	1	0	0	0
Combined	**52**	**42**	**6**	**0.787±0.026**	**0.028±0.003**	**12.587**

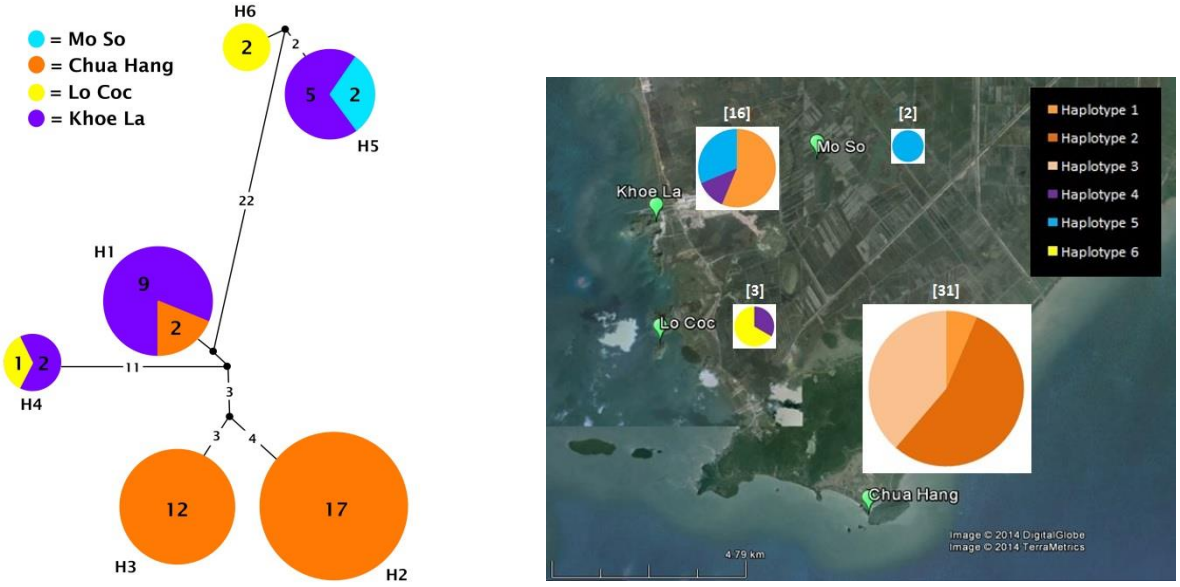


Figure 3.2. Haplotype network and haplotype distribution of Indochinese silvered langurs. Median-joining network depicting haplotype relationships is on the left. Circle sizes are proportional to haplotype frequency (the smallest circle represents 2 samples, and the largest circle represents 17 samples). Each node (small black circles) represents missing intermediate haplotype. The number on each vector line accounts for the number of mutational steps (vector with no number represents one mutational step between the two haplotypes). The map on the right shows the haplotype frequencies (pie charts) within each site/population. Each color represents one of the 6 haplotypes. The chart area is scaled to the sample size (also in brackets).

Haplotype H2 had the highest frequency (33%), followed by H3 (23%), the second most frequent. Both H2 and H3 were exclusive to Chua Hang hill. This distribution likely reflected the joint effects of a sampling bias because Chua Hang hill encompassed 60% of the total Indochinese silvered langur samples, the low number of haplotypes, and the lack of recent gene flow between the isolated subpopulations. Only Khoe La hill, the site under the most intense anthropogenic disturbance through mining activities of the four sites, contained haplotypes that were also found in the other sites (Fig. 3.2).

The Indochinese silvered langur is a flagship species of Kien Luong Karst Area in Kien Giang Province, home to the largest population in Vietnam (Hoang Minh Duc *et al.* 2010c). It is estimated that there are 286 individuals left in the wild here, of which about 40% are found within Khoe La hill (Tran Van Bang *et al.* submitted). Limestone quarrying

fragmented Khoe La hill into two sections, dividing and isolating the silvered langurs into subpopulations in the north and south. Mining activities in the north also continue to shrink the habitat, significantly restricting population movement and decreasing food resources. As the subpopulation in north Khoe La is the only one containing haplotypes that can also be found in the other three hill sites in the Karst Area, local extirpation of this subpopulation will imply that the current gene pool can no longer be mixed given that each of the other three populations does not share haplotypes. This demonstrates an added importance of the north Khoe La subpopulation for preserving total genetic diversity of the species in the Karst Area. Immediate action to move the Indochinese silvered langurs out of north Khoe La into south Khoe La, where no mining activities are currently scheduled, is necessary (Fig. 3.3). Given that active translocation is often risky (especially for unhabituated and arboreal Indochinese silvered langurs inhabiting karst hills) and may result in the death of the animals during capture and transport, installing rope ladders between north and south Khoe La, and eventually to Hang Tien hill (where no mining activity is taking place and where no langur population has been reported) can be explored to restore wildlife movement and reestablish genetic connectivity between the populations.

Figure 3.3. Limestone hills of north Khoe La, south Khoe La, and Hang Tien, where rope ladders can be installed to connect them (Photo: Andie Ang).

One of the earliest recorded installations of canopy bridges for primate conservation was in 1991 across a black lion tamarin (*Leontopithecus chrysopygus*) and tufted capuchin

(*Sapajus apella*) road traffic mortality hotspot in Fazenda Rio Claro, Brazil (Valladares-Padua *et al.* 1995). They employed two parallel wooden poles stretched above the road. In 1997, string bridges were erected across a vervet monkey (*Chlorocebus pygerythrus*), Sykes monkey (*Cercopithecus albogularis*) and Angola colobus (*Colobus angolensis*) road traffic mortality hotspot in Diani, Kenya (www.colobusconservation.org). More recently, Teixeira *et al.* (2013) reported on the use of canopy bridges by brown howler monkey (*Alouatta guariba clamitans*) in Porto Alegre, Brazil.

Figure 3.4. Exploring north Khoe La during a site visit in 2015 with Dr. Herbert Covert and Dr. Hoang Minh Duc, with the help of Holcim Ltd (Photo: Andie Ang).

Together with the Southern Institute of Ecology (SIE) of the Vietnam Academy of Science and Technology (VAST), who conducts biodiversity research and conservation in the southern provinces (including the Kien Luong Karst Area), we have received a National

Geographic Waitt Grant to install rope ladders and explore their effectiveness in the conservation of the Indochinese silvered langurs in Khoe La. In June 2015, Dr. Herbert Covert, scientific advisory committee member to SIE, Dr. Hoang Minh Duc, vice-director of SIE, and I presented this project to the People's Committee of Kien Luong district of Kien Giang Province and the Department of Agriculture and Rural Development of Kien Giang Province. A working agreement has also been reached with the Swiss-based building and mining company Holcim Ltd., which is quarrying north Khoe La to cooperate with the canopy bridge installation and reforestation of the area to create green corridor (Fig. 3.4). Once natural and artificial green corridors are created, the langurs would have higher breeding opportunities, greater resource availability, and hence an elevated probability of population survival in the future.

3.7. Genetic Variability of the Tonkin Snub-nosed Monkey

I collected 254 fecal samples of Tonkin snub-nosed monkeys (~2x population size in Khau Ca Area); i.e., most of the snub-nosed monkeys in Khau Ca Area were probably sampled with some being analyzed several times. Two hundred and one HV-I sequences (467-650bp) were obtained using Sanger (483-650bp) and NGS sequencing (467bp) and all sequences were identical for the overlapping region (461bp) (Hypothesis 3a). They have been deposited into GenBank with accession numbers KX609035 – KX609235. Shotgun sequencing of five samples (T78, T105, T110, T135, T191) generated 104-142 million high quality sequences per sample, whereby mapping to reference mitochondrial genome led to average coverage of 5.1X-41.2X. SNP calling did not identify any variable sites. Note that this is the lowest mitochondrial genetic variability ever reported for any primate species in the wild (Hypothesis 3b) (Tables 3.1 and 3.7).

This lack of variability is unlikely the result of contamination because (1) each fecal sample was stored in individual sterile vials, (2) extractions were performed on randomly

chosen samples on different days on freshly cleaned lab benches, (3) results were identical for different extraction methods, (4) results were identical for Sanger and NGS barcodes, (5) no cross-contamination was observed with other samples (i.e. of black-shanked doucs and Indochinese silvered langurs) in the same NGS run and (6) randomly selected samples for mitochondrial genomes gave same results as HV-I, even though processed independently. When compared to available sequences in GenBank, the unique haplotype for Khau Ca population was 98.29% identical to HM125578.1 (Yu *et al.* 2011, Eastern Vietnam) and 97.64% identical to both JF293093.1 (Roos *et al.* 2011, Endangered Primate Rescue Center, EPRC, Vietnam) and EU004480.1 (Osterholz *et al.* 2008, EPRC). Unfortunately, specific locality information of these samples is not available.

The Tonkin snub-nosed monkey population in Khau Ca Area is arguably the most important population for the survival of the species because the habitat is better protected, the population is continuously monitored, and conservation education programs are in place (Le Khac Quyet *et al.* 2015). It is also by far the largest population and infants have been observed for the last 10 years (Le Khac Quyet, personal communication). Although the Khau Ca population appears to be recovering, the absence of any variability for the HV-I region, which is among the most rapidly evolving part of the fast mitochondrial genome (Lopez *et al.* 1997), is a major concern. This lack of variability was not restricted to the HV-I region, but extended to the entire mitochondrial genome as documented via shotgun sequencing of fecal samples. The low genetic variability of Tonkin snub-nosed monkey is thus more similar to another recovering Southeast Asian primate population; i.e., Singapore's Raffles' banded langur (*Presbytis femoralis femoralis*) which was believed to be on the brink of extinction with <30 individuals left in the 1990s (Ang *et al.* 2012). The population size has approximately doubled (40-60), but it also shows extremely low variability at the HV-I region (Ang *et al.* 2012). The after-effects of bottlenecks in the 1970s and 1980s are clearly

recognizable which is likely to make the population extremely vulnerable to environmental change (e.g. disease, Spielman *et al.* 2004).

Low genetic variability of the Tonkin snub-nosed monkey in Khau Ca Area is likely the result of widespread deforestation and intensive hunting in recent decades which severely fragmented the habitat (Nadler 2014), leading to population bottlenecks and restricted gene flow (the closest other population in Tung Vai Forest is 35 km away and separated by highly modified landscapes). While some species with low genetic variation appears to be able to persist (e.g. black-footed ferrets, Wisely *et al.* 2002; African cheetahs, Castro-Prieto *et al.* 2011), loss of genetic variation is also associated with increased extinction risk (e.g. Tasmanian devil, Miller *et al.* 2011).

Low diversity in maternally inherited mtDNA may not reflect total genetic variation (Kolleck *et al.* 2013) and nuclear markers should ideally be included. However, they are difficult to develop and in this case primers for closely-related species failed to reliably amplify when applied to the DNA that can be obtained from fecal samples. The lack of success may be partially due to the low concentration of primate endogenous DNA in fecal samples (0.05-0.68% of total DNA, Table 3.6). However, my finding that there was a complete lack of genetic variability in the mitochondria means that the development of nuclear markers is now a priority. Both mitochondrial and nuclear markers should then be examined for other populations, especially Tung Vai population of ca. 20 Tonkin snub-nosed monkeys in order to assess remaining genetic diversity of the species. The presence of two additional HV-I haplotypes in GenBank and four mitochondrial cytochrome b haplotypes in Roos *et al.* (2007; locality information is not available) suggests that the overall genetic variability in the species may be higher.

Table 3.6. Estimation of amount of endogenous primate nuclear DNA in Tonkin snub-nosed monkey fecal metagenomes. Paired reads were mapped onto *Rhinopithecus roxellana* draft assembly (6,205 scaffolds)

Sample	Total number of reads	Number of paired reads	Number of mapped reads	Number of mapped reads <5bp from reference
T78	129,446,297	119,925,292	68,194 (0.06%)	61,689 (0.05%)
T110	104,284,677	95,917,160	185,320 (0.19%)	170,214 (0.18%)
T105	142,294,641	132,965,282	165,428 (0.12 %)	151,706 (0.11%)
T135	130,084,972	124,931,604	928,395 (0.74%)	855,600 (0.68%)
T191	107,305,258	102,372,058	83,758 (0.08%)	75,615 (0.07%)

3.8. Summary

This chapter presents the first information on the mitochondrial genetic variability of three species of colobine primates in Vietnam: the black-shanked douc (*Pygathrix nigripes*; BSD), Indochinese silvered langur (*Trachypithecus germaini*; ISL), and Tonkin snub-nosed monkey (*Rhinopithecus avunculus*; TSNM). Mitochondrial genetic variability was assessed using the hypervariable region I of d-*loop*. Thirty-seven HV-I sequences were obtained from the BSD populations in Ta Kou Nature Reserve, Cat Tien National Park, Nui Chua National Park, and Hon Heo Mountain. When examined together, the species exhibited high genetic variability. Interestingly, there were no shared haplotypes between the four sites, indicating that recent gene flow between all the populations has been restricted. Fifty-two HV-I sequences of ISLs in the Kien Luong Karst Area showed relatively high genetic variability, but when considered separately, each of the four subpopulations exhibited low variability. Only Khoe La hill, the site under the most intense anthropogenic disturbance through mining activities of the four sites, contained haplotypes that were also found in the other sites. Extirpation of the Khoe La subpopulation would mean that all remaining haplotypes would become isolated from each other. Therefore, it is imperative that the ISLs from Khoe La hill are moved to other karst hills, either via active translocation or by creating eco-corridors to facilitate movement. Lastly, a total of 201 fecal samples from the TSNM population in the

Khau Ca Area was sequenced and they showed zero variability at the HV-I region. A subset of five samples analyzed via shotgun sequencing also showed no variable sites in the entire mitochondrial genomes. This result highlights the immediate need for a comprehensive assessment of the genetic diversity in all remaining populations of TSNMs based on both mitochondrial and nuclear markers. The latter needs to be developed for this species and also for the BSDs and ISLs.

Table 3.7. Genetic variability (HV-I/d-*loop*) of colobine primates from major published studies and this study

Species	**Sample Location**	**N**	**Bp**	**V**	**H**	***h***	***π***	**Π**	**Reference**
Angolan black and white colobus* (*Colobus angolensis palliatus*)	Diani, Shimoni [KE] Udzungwa, Rungwe [TZ]	103	1,795	101	19	0.64-0.85± 0.034-0.095	0.0021-0.0113± 0.001-0.006	NA	McDonald & Hamilton 2010
Western black-and-white colobus (*C. polykomos*)	Cantanhez [GW]	56	478	2	3	0.17±0.065	0.00038±0.0001	0.17	Minhós *et al.* 2013
Proboscis monkey (*Nasalis larvatus*)	Sabah [MY]	29	264	21	16	0.90	0.022	NA	Munshi-South & Bernard 2011
Raffles' banded langur (*Presbytis femoralis femoralis*)	Central Catchment [SG]	7	422-522	1	2	NA	NA	NA	Ang *et al.* 2012
Temmink's red colobus (*Procolobus badius temminckii*)	Cantanhez [GW]	79	448	45	9	0.82±0.017	0.037±0.002	16.5	Minhós *et al.* 2013
Black-and-white snub-nosed monkey (*Rhinopithecus bieti*)	Yunnan/Tibet [CN]	157	401	51	30	0.945±0.006	0.036±0.018	13.773±6.216	Liu *et al.* 2007
Gray snub-nosed monkey (*R. brelichi*)	Fanjingshan [CN]	141	603	25	5	0.457±0.048	0.014±0.007	5.259±2.557	Yang *et al.* 2012
Golden snub-nosed monkey (*R. roxellana*)	Sichuan/Gansu/ Shaanxi/Hubei [CN]	60	379	54	12	0.845±0.026	0.034±0.017	13.029±5.949	Li *et al.* 2007
Delacour's langur (*Trachypithecus delacouri*)	Van Long/Kim Bang [VN]	70	390	7	4	0.424±0.066	0.003±0.002	1.224±0.788	Ebenau *et al.* 2011
White-headed langur (*T. leucocephalus*)	Guangxi [CN]	77	574	10	9	0.570±0.056	0.00323±0.00044	NA	Wang *et al.* 2015
Black-shanked douc (*Pygathrix nigripes*)	**Ta Kou/Cat Tien/Nui Chua/Hon Heo [VN]**	**37**	**461-1,093**	**52**	**14**	**0.896±0.033**	**0.032±0.003**	**14.565**	**This study**
Indochinese silvered langur (*T. germaini*)	**Kien Luong Karst Area [VN]**	**52**	**455-1,091**	**42**	**6**	**0.787±0.026**	**0.028±0.003**	**12.587**	**This study**
Tonkin snub-nosed monkey (*R. avunculus*)	**Khau Ca [VN]**	**201**	**467-650**	**0**	**1**	**0**	**0**	**0**	**This study**

N: number of individuals/samples; Bp: number of base-pairs; V: number of polymorphic sites; H: number of haplotypes; h: haplotype diversity; π: nucleotide diversity; Π: average number of nucleotide differences. *The 1,795bp sequences included 1,140bp of cytochrome b, the first 524bp of d-*loop*, along with 18bp of mRNA and 113bp of tRNAs.

CHAPTER IV
DIET METABARCODING

4.1. Introduction

While much can be learned about primates by means of observation (e.g. for social behavior), the slow life history of many primates and the difficulty in collecting long-term data mean that decades of dedicated effort is often not enough to illuminate long-term evolutionary processes (Vigilant & Guschanski 2009). Furthermore, many threatened animals are rare and elusive, and live in remote and inaccessible habitats which prohibit directly collecting data on species' biology such as mating patterns, reproductive systems, and social structures (Kuhl *et al.* 2008). Collecting ecological data on primates in the field has also been noted to be challenging and requires a long study period (e.g. Chivers & Raemaekers 1980; Woodruff 2004; Ang 2010; Grueter *et al.* 2010; Workman & Le Van Dung 2010; Kappeler *et al.* 2012). Many food plants particularly rarer ones may also be missed out because it is difficult to locate or follow the animals. In this case, fecal samples become important in complementing field observational data as they contain ecological information even without direct observation (Kohn & Wayne 1997; Ferreira da Silva *et al.* 2012). DNA-based methods using fecal samples are able to provide accurate information on host genetics, diet, gut parasites and intestinal flora of a species (e.g. Lamendella *et al.* 2011; Ferreira da Silva *et al.* 2012; Shehzad *et al.* 2012).

The emergence of next-generation sequencing (NGS) technologies in association with DNA metabarcoding has recently made possible the performance of rapid diet analysis by identifying DNA sequences of consumed items from fecal material by directly sequencing in parallel thousands of short DNA barcodes amplified using universal primers (see Taberlet *et al.* 2012). While Ang (2010) documented 23 food plant species from 17 families based on direct observation of Raffles' banded langurs (*Presbytis femoralis femoralis*) for 12 field

months, Srivathsan *et al.* (2016) employed DNA metabarcoding on fecal samples collected in Ang (2010) to retrieve at least 53 plants species from 33 families.

The dietary profiles for several tens or hundreds of individuals of one species or several species can also be obtained simultaneously, generating an understanding on the foraging ecology at a population level, especially if fecal material can be collected across seasons and habitats (e.g. Shehzad *et al.* 2012 for snow leopards; Hibert *et al.* 2013 for lowland tapirs; Quéméré *et al.* 2013 for golden-crowned sifakas; de Barba *et al.* 2014 for brown bears; Lopes *et al.* 2015 for rodents). Combining DNA metabarcoding with next-generation sequencing, Quéméré *et al.* (2013) identified 130 food plants belonging to 80 genera from fecal samples collected in the dry season of critically endangered golden-crowned sifakas (*Propithecus tattersalli*), which appeared consistent with the number obtained by Meyers (1993) who recorded 153 food species in 88 genera from direct field observation over both dry and wet seasons. Moreover, the list from Quéméré *et al.* (2013) contained 47 new genera not recorded during field observation in Meyers (1993). This additional information complements direct observation in characterizing dietary profiles of animals, and is especially useful if the animals are elusive and difficult to follow (Hibert *et al.* 2013). In a diet metabarcoding of dung piles of lowland tapirs (*Tapirus terrestris*) in French Guiana, Hibert *et al.* (2013) demonstrated the efficacy of this approach in accurately identifying food species of an elusive herbivore: 95% (39 of 41) and 74% (29 of 39) of the plant families and genera identified respectively using metabarcoding were already known to be consumed by tapirs based on direct observation (Hibert *et al.* 2011). Furthermore, this approach identified two plant families and seven genera not known to be consumed by the lowland tapir, lending support to the use of diet metabarcoding in complementing classical approaches of studying diet of an elusive mammal (Hibert *et al.* 2013).

Diet metabarcoding is not without limitations; for instance, more research is necessary before sequence read counts obtained can directly be correlated with biomass intake/feeding preference (Srivathsan *et al.* 2015). This limitation may be due to various factors, such as amplification biases towards certain taxa during PCR (Pompanon *et al.* 2012), differential rates of digestion (Deagle *et al.* 2010) and structural differences between fruits and leaves consumed (Srivathsan *et al.* 2015). Furthermore, high variability in plant taxa among fecal pellets collected from the same individual at the same time was found in the Pacific pocket mouse, which may be due to consumption patterns and/or differential digestion (Iwanowicz *et al.* 2016). This fecal heterogeneity can affect recovered diet components if only parts of each fecal sample or only one sample per animal were examined.

Despite some of these complications related to diet metabarcoding, this DNA-based approach enables simultaneous and large-scale identifications such as food plants to species level and provides information on food plant diversity which allows us to examine various other aspects of feeding strategies. Here, I aim to characterize and evaluate the dietary profiles of the black-shanked douc, Indochinese silvered langur, and Tonkin snub-nosed monkey at various taxonomic levels and in relation to (1) feeding observations from the field, and (2) floristic diversity within their habitats. These results are also discussed in light of population differences, effects of habitat disturbances, and diet transition between seasons.

4.2. DNA Extraction

Genomic DNA was extracted from the interiors of fecal samples so as to minimize the contamination from environmental DNA such as pollen or leaf litter. A total of 294 fecal samples (37 from black-shanked doucs, 40 Indochinese silvered langurs, and 217 Tonkin snub-nosed monkeys) and one positive control consisting of sapodilla (*Manilkara zapota*; Sapotaceae), papaya (*Carica papaya*; Caricaceae), and banana (*Musa* sp.; Musaceae) were extracted using standard phenol-chloroform extraction (following Kutty *et al.* 2007). For each

sample, a piece of ca. 50mg feces/fruits was used (see **Chapter 3.2. on DNA Extraction** for extraction procedure and DNA storage). Standard stool kit such as from Qiagen was not used because it is known to contain starch compounds which will interfere with diet metabarcoding results.

4.3. DNA Amplification and Sequencing

For diet analyses, the P6 loop of plant chloroplast trnL intron from fecal DNA and positive control were amplified using primers trnL-g and trnL-h (following Taberlet *et al.* 2007). Each sample was tagged with eight variable nucleotides designed by Srivathsan *et al.* (2015) at the 5' end of each primer (Coissac *et al.* 2012; ≥5 variable sites; <3 bp homopolymers; additional dinucleotide CC was added to 5' end). PCR amplifications were carried out for 45 cycles as in Quéméré *et al.* (2013) using the same mixture proportions as for HV-I amplification (see **Chapter 3.3. DNA Amplification and Sequencing**). Two independent PCR replicates were obtained for each fecal sample. Additionally, two independent PCR replicates were obtained for the positive control with known concentrations: *Manilkara zapota* (Sapotaceae) 2.02ng/µl, *Carica papaya* (Caricaceae) 14.4ng/µl and *Musa* sp. (Musaceae) 1.74ng/µl. The resulting amplification products were electrophoresed on 4% agarose gels, and visualized by GelRed (Biotium) staining. All PCR products were then purified using MinElute PCR Purification Kit (QIAGEN) and sequenced on NGS Illumina MiSeq (Nano kit 2x150PE) after preparing two TruSeq libraries (August 2015) for amplicons (one replicate in each library).

4.4. Data Analysis

4.4.1. Sequence Analysis

In order to obtain a dietary profile of the samples based on metabarcoding, I adapted published metabarcoding analysis pipelines (Quéméré *et al.* 2013; Srivathsan *et al.* 2015). First, the forward and reverse reads were merged using PEAR (version 0.9.1) (Zhang *et al.*

2014). The merged reads were then analyzed using OBITools and associated programs (Boyer *et al.* 2016). Sequences were assigned to their individual samples based on the primers and unique identifier tags with "ngsfilter" program. Only sequences with identical match on tags and a maximum of two errors on primers were kept. After removing the primer and tag sequences, identical trnL sequences were clustered using "obiuniq". Sequences <=10 bp were excluded using "obigrep". Then, the sequences were assigned 'head', 'singleton', and 'internal' using "obiclean". 'Head' indicates that this sequence is the most common sequence among all sequences that can be linked with a single indel or substitution; 'singleton' means that there is no other variant in that sample; and 'internal' refers to all other sequences not being 'head' or 'singleton', corresponding to amplification or sequencing errors (Shehzad *et al.* 2012).

I used the positive and negative controls included in the experiment to set two additional filtering parameters: 1) I determined a threshold based on positive controls such that upon applying this threshold the positive controls retained only relevant sequences and noisy sequences were discarded. This threshold was used across all the samples in a given library (de Barba *et al.* 2014). Sequences assigned as 'internal' were then removed and only 'head' and 'singleton' sequences were retained. 2) A matrix of pairwise distances among sample replicates was created computing the Renkonen Similarity Index, RE (Renkonen 1938) based on sequence frequency data:

" $\mathrm{RE} = \sum_{i=1}^{n} min(p_i Library\ 1\ ,\ p_i Library\ 2)$ ",

where i = sequence, n = total number of sequences, min ( ) = the smaller of the two numbers within ( ) and p_i = frequency of sequence i

Distance measures took a maximum value of 1 if the sets of relative abundances compared were identical and a minimum value of 0 for completely distinct assemblages (no sequences in common). I then used the RE value of the pair of negative replicates from libraries 1 and 2 as a threshold. Fecal sample replicates with RE values lower than the threshold were

discarded. Sequences not present in both sample replicates were also removed. Finally, each of the filtered sequences was matched against the global and local databases in order to assign a taxon to them.

4.4.2. Statistical Analysis

The One-Way Analysis of Variance (ANOVA) was used to determine whether there were any significant differences between the average number of sequences detected in fecal samples collected from different sites, and post-hoc tests were calculated using the Tukey test. ANOVA was only applied for the black-shanked douc (four sites) and Indochinese silvered langur (four sites) samples, and not on the Tonkin snub-nosed monkey samples (one site). All statistical analyses were performed using SPSS Statistics 24.

4.4.3. Taxonomic Reference Databases

A global taxonomic reference database was built by extracting all the sequences of the trnL intron available for Magnoliophyta (angiosperms) in GenBank using the "ecoPCR" program of OBITools (Boyer *et al.* 2016). All GenBank sequences matching to "trnL", "trnL-F", or "trnL-trnF" were downloaded, generating a database containing 222,238 sequences. Identifications of the diet sequences were made using "ecotag" in which a family/genus name is assigned if the sequence identity is ≥95% to the database and species name if the identity is ≥98% (de Barba *et al.* 2014). However, upon inspecting the best identity assignments, a portion of the sequences either did not have an identity match, were assigned to taxa (family/genus level) with less than 95% identity score, were assigned to a species not known to occur in Vietnam, or were assigned to a higher taxonomic level than family. This is likely due to two main reasons: 1) The P6 loop of trnL has a small size which makes it suitable for analyzing diet from degraded DNA (Pompanon *et al.* 2012) but it has a low taxonomic resolution at the species level; 2) there is a lack of trnL sequences from taxa found within the

habitats of the three primate species in the GenBank database (Table 4.2; list of plant taxa not in GenBank under Appendix A).

In order to refine the taxonomic assignation, I generated a local database using the lists of plant taxa (genus and species levels) that are known to occur within the habitats of the three primate species (specifically within the study sites) and retrieved their corresponding trnL sequences (following the method of Quéméré *et al.* 2013). For the black-shanked douc (BSD), Indochinese silvered langur (ISL), and Tonkin snub-nosed monkey (TSNM), I used the flora checklists of Ta Kou Nature Reserve (Luu Hong Truong 2000, 2006) and Cat Tien National Park (Cat Tien National Park 2012), Kien Luong-Kien Hai forests (see Nguyen Xuan Dang 2009), and Khau Ca Area (Vu Anh Tai *et al.* 2013) respectively. Table 4.1 summarizes the plant diversity within these sites based on the studies. A total of 98,855 sequences were retrieved and after clustering identical taxa, 15,849 unique sequences remained, forming the local database to which the final list of sequences comprising the fecal samples was queried.

Table 4.1. Plant diversity within the study sites of the three primate species.

Primate species/Study site	**# of families**	**# of genera**	**# of taxa**	**Reference**
Black-shanked douc (BSD)				
Ta Kou Nature Reserve	117	365	595	Luu Hong Truong 2000, 2006
Cat Tien National Park	167	697	1,607	Cat Tien National Park 2012
Indochinese silvered langur (ISL)				
Kien Luong-Kien Hai	156	563	927	Nguyen Xuan Dang 2009
Tonkin snub-nosed monkey (TSNM)				
Khau Ca Area	127	346	532	Vu Anh Tai *et al.* 2013

*Numbers reported here vary from the original articles after I updated the flora lists using The Plant List 2013a.

Table 4.2. Proportion of plant genera and taxa within study sites that did not have trnL sequences in GenBank.

Sites	**Proportion of genera (%) not in GenBank**	**Proportion of taxa (%) not in GenBank**
Cat Tien	194/697 (27.8)	n/a
Ta Kou	93/365 (25.5)	441/595 (74.1)
Kien Luong	90/563 (16.0)	569/927 (61.4)
Khau Ca	52/346 (15.0)	317/532 (59.6)

4.4.4. Diet Metabarcoding Dataset

Two replicates of each of the 294 fecal samples (37 from BSD, 40 ISL, and 217 TSNM), one positive control, and one negative control totaling to 592 samples were sent for next-generation sequencing. Table 4.3 summarizes the number of samples and sequences retained after each filtering step. A total of 5,406,020 paired-end sequence reads (98.8% total reads) for library 1 and 6,013,911 (98.5%) for library 2 were obtained. After applying the OBITools programs, I retained 37,537 unique sequences (or MOTUs for Molecular Operational Taxonomic Units) with a total count of 3,500,316 reads (64.0% of the initial total count) for library 1 and 40,309 unique sequences with a total count of 4,338,405 reads (71.0%) (Fig. 4.1a).

Initial inspection of positive amplifications revealed that sequences corresponding to the three plant families composing the positive control mix (Sapotaceae, Caricaceae, and Musaceae) were detected. Most other sequences (due to amplification or sequencing errors) had a much lower frequency of occurrence compared to these known sequences. The positive control samples would retain all relevant sequences and be cleaned of all noisy sequences after applying a threshold frequency of occurrence of 0.00392 for library 1 and 0.00360 for library 2. I used this observation to identify and remove sequences with a per sample frequency of occurrence lower than 0.00392 and 0.00360 for each fecal sample in library 1 and library 2 respectively. At this point, 220, 149, and 98 unique sequences were retained for BSD, ISL, and TSNM samples respectively (Fig. 4.1b). After removing sequences assigned as ‘internal’ and keeping only ‘head’ and ‘singleton’ sequences, 173, 111, and 65 unique sequences were retained (Fig. 4.1c).

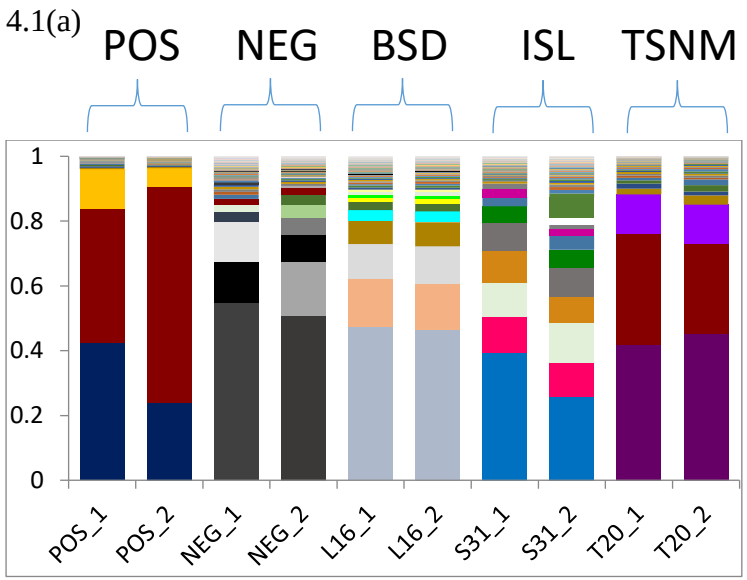
As expected, the two negative replicates in library 1 and library 2 showed a greater number of highly dissimilar sequences in contrast to the positive controls and fecal sample replicates due to the amplification of non-specific PCR products in the negative controls.

Figure 4.1d illustrates the distributions of Renkonen similarity index between replicates of positive and negative controls and all fecal samples (a table of RE values in Appendix B). The RE value for the pair of negative replicates is 0.131. Therefore I discarded fecal samples whose RE values between replicates are lower than 0.131 (Fig. 4.1e). Only one sample (L81, black-shanked douc, RE = 0.039) has a RE value <0.131 and was removed. After removing sequences not found in both replicates, 110, 68, and 32 unique sequences were retained for BSD, ISL, and TSNM samples respectively (Fig. 4.1f). Each of the diet sequence was given a plant identification wherever possible using the global and local databases. The identifications were also manually checked for inaccurate identifications, such as to species or genera not known to be found in the study sites or Vietnam (Fig. 4.1g). Diet metabarcoding results specific for each of the three primate species are presented and discussed within their respective chapters (Chapters V, VI, and VII).

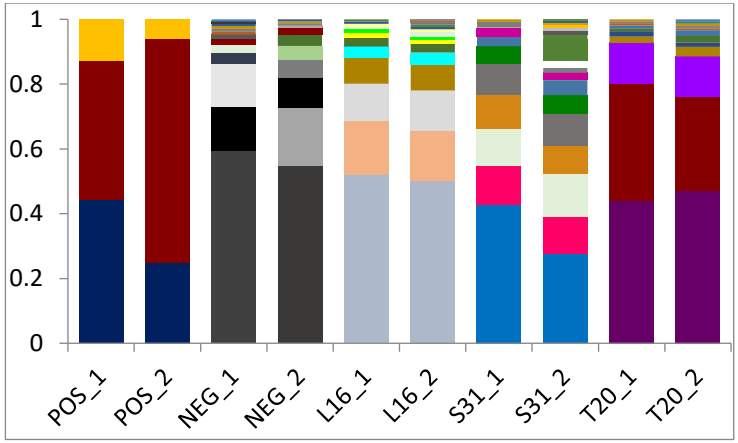
Table 4.3. Summary of the number of sequences and samples (shaded rows) after different steps of the data filtering steps for the 1 positive, 1 negative, 37 BSD, 40 ISL, 217 TSNM, 2 replicates per sample, 1 replicate in each library. The proportion of sequences remaining is indicated in parenthesis.

	Library 1	Library 2	Figure
Total reads	5,469,094 (100%)	6,107,676 (100%)	n/a
Assembled paired-end sequence reads (forward and reverse reads merged)	5,406,020 (98.8%)	6,013,911 (98.5%)	n/a
Sequence reads for which primers and tags were identified	3,500,316 (64.0%)	4,338,405 (71.0%)	n/a
Unique sequences at beginning of analysis	37,537	40,309	4.1a
Samples at beginning of analysis (+/–/BSD/ISL/TSNM)	1/1/37/40/217	1/1/37/40/217	n/a
Unique sequences after removal of low-frequency noise based on positive control (+/–/BSD/ISL/TSNM)	3/21/220/149/98		4.1b
Unique sequences after removal of sequences not identified as 'head' or 'singleton' (+/–/BSD/ISL/TSNM)	3/16/173/111/65		4.1c
Samples after removal of unreliable amplifications based on RE value (+/–/BSD/ISL/TSNM)	1/0/36/40/217		4.1e
Unique sequences after removal of sequences not present in both sample replicates (+/–/BSD/ISL/TSNM)	3/0/110/68/32		4.1f
Unique sequences after creating consensus sequence profiles (+/–/BSD/ISL/TSNM)	3/0/110/68/32		4.1g
Final fecal samples (BSD/ISL/TSNM)	36/40/217		n/a

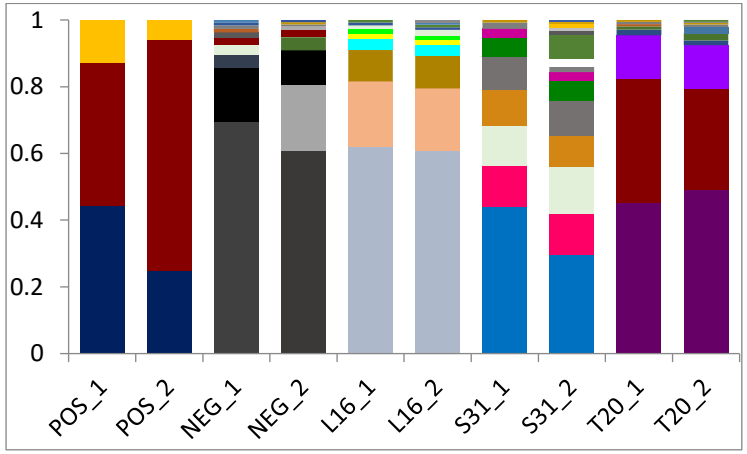
4.1(a)


POS
NEG
BSD
ISL
TSNM
1
0.8
0.6
0.4
0.2
0
POS_1
POS_2
NEG_1
NEG_2
L16_1
L16_2
S31_1
S31_2
T20_1
T20_2

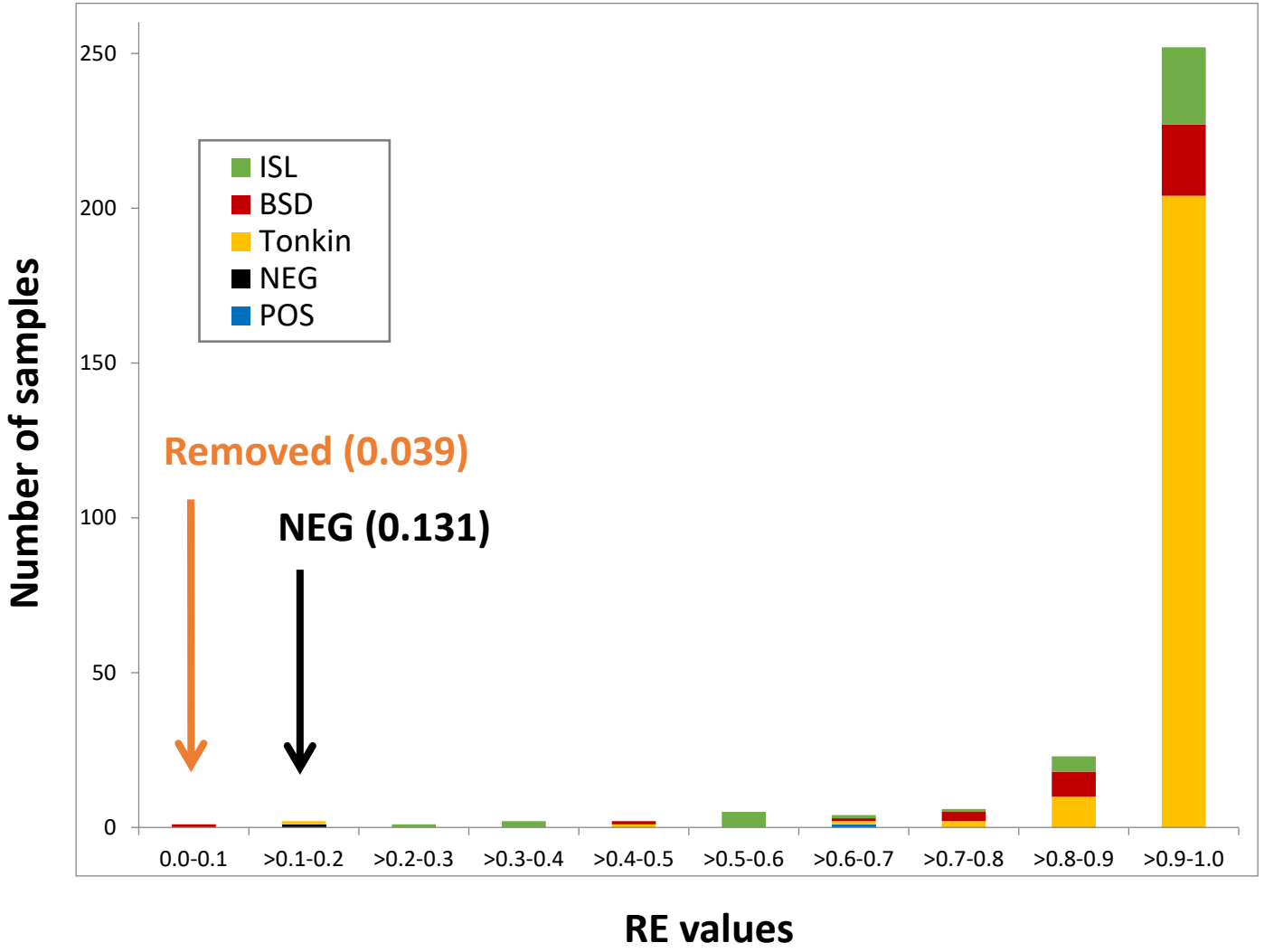
(b)


1
0.8
0.6
0.4
0.2
0
POS_1
POS_2
NEG_1
NEG_2
L16_1
L16_2
S31_1
S31_2
T20_1
T20_2

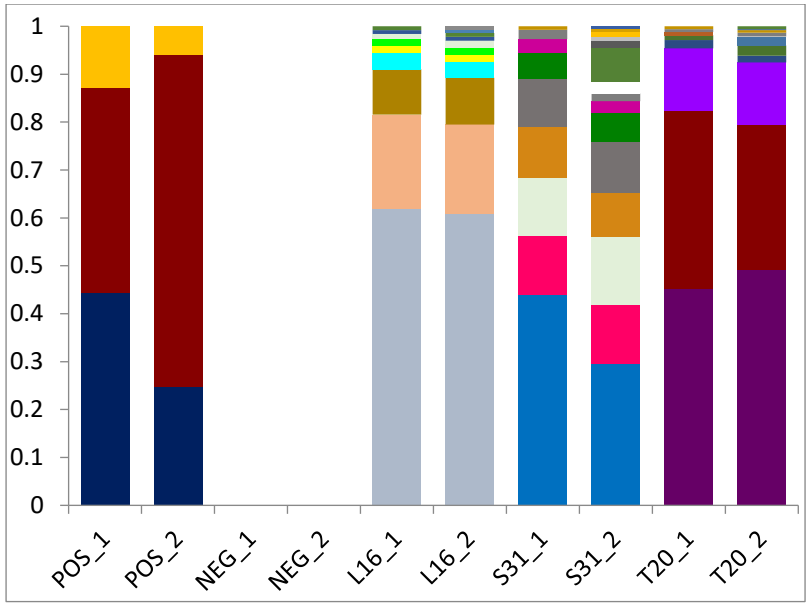
(c)


1
0.8
0.6
0.4
0.2
0
POS_1
POS_2
NEG_1
NEG_2
L16_1
L16_2
S31_1
S31_2
T20_1
T20_2

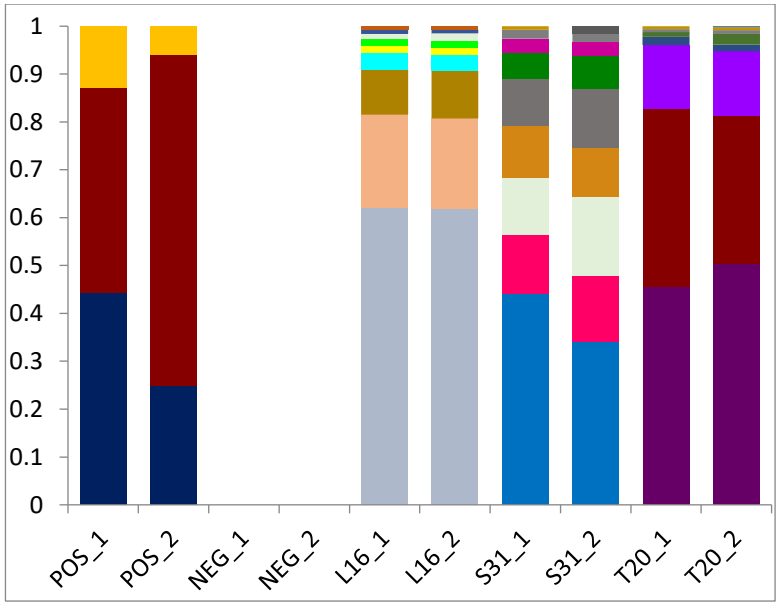
(d)


250
200
150
100
50
0
Number of samples
ISL
BSD
Tonkin
NEG
POS
Removed (0.039)
NEG (0.131)
0.0-0.1
>0.1-0.2
>0.2-0.3
>0.3-0.4
>0.4-0.5
>0.5-0.6
>0.6-0.7
>0.7-0.8
>0.8-0.9
>0.9-1.0
RE values

(e)


1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
POS_1
POS_2
NEG_1
NEG_2
L16_1
L16_2
S31_1
S31_2
T20_1
T20_2

(f)


1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
POS_1
POS_2
NEG_1
NEG_2
L16_1
L16_2
S31_1
S31_2
T20_1
T20_2

(g)

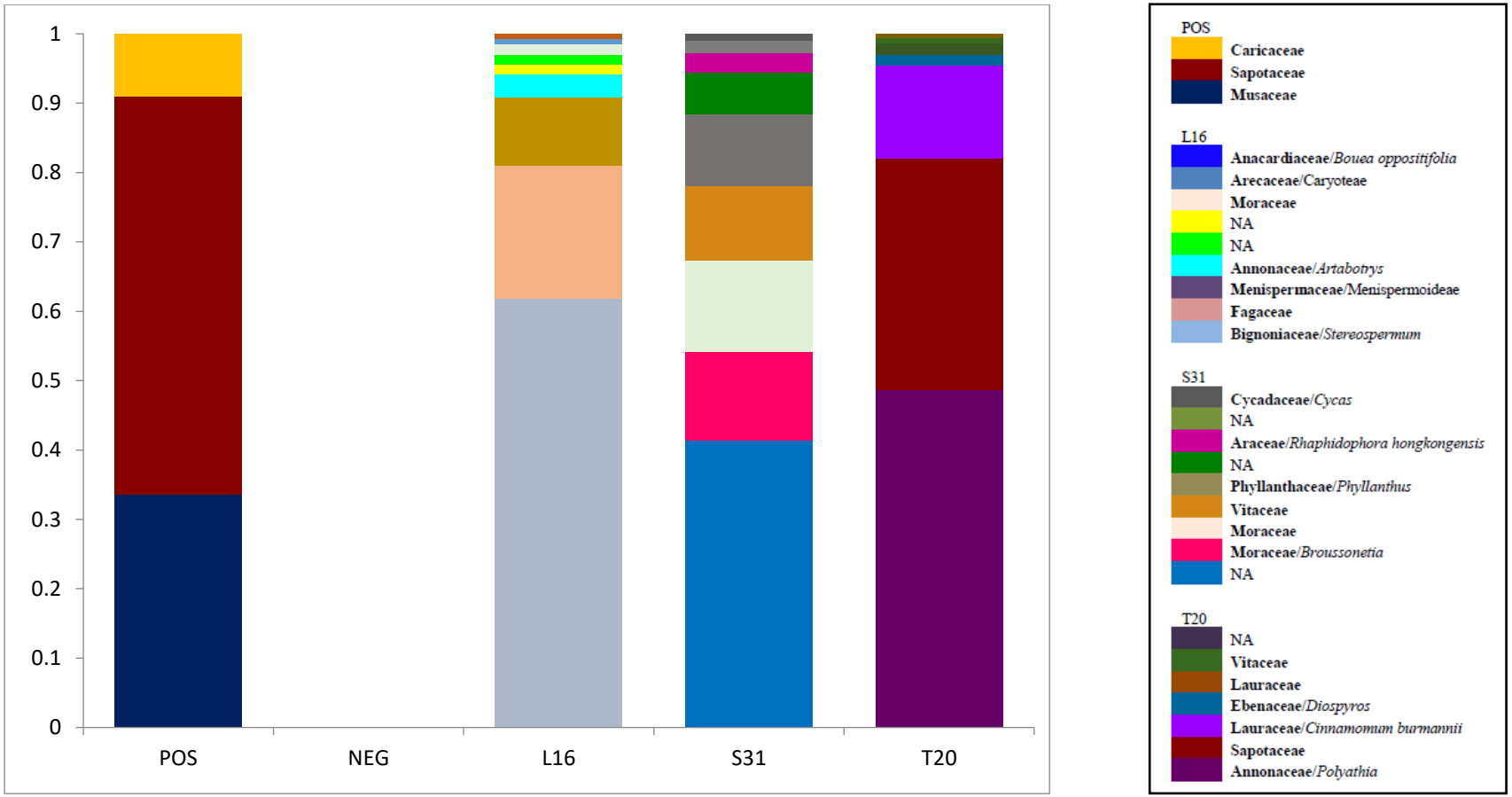


Figure 4.1. Left-Graphical example of subsequent steps of the second part of filtering process, for replicates of positive (POS_1, POS_2), negative (NEG_1, NEG_2) controls, and two replicates of each of three fecal samples (L16 for black-shanked douc, S31 for Indochinese silvered langur, T20 for Tonkin snub-nosed monkey). Colored bars represent amplification products, with colors denoting different sequences retained after each filtering step and their frequency. (a) Beginning of analysis; (b) removal of low-frequency noise based on threshold given by POS; (c) removal of erroneous sequences identified using obiclean (retaining only 'head' and 'singleton'); (d) distributions of Renkonen similarity index between replicates; (e) removal of samples with unreliable replicates (based on RE value 0.131); (f) removal of sequences not found in both replicates; (g) construction of consensus sequences profiles. Right-Legend showing identifications corresponding to each of the colored bars.

CHAPTER V

DIETARY PROFILE OF BLACK-SHANKED DOUCS

5.1. Introduction

The genus *Pygathrix* E. Geoffroy Saint-Hilaire 1812 contains three extant species, namely the red-shanked douc (*P. nemaeus*), grey-shanked douc (*P. cinerea*), and black-shanked douc (*P. nigripes*) which are distributed on the Indochina Peninsula in Vietnam, Cambodia, and Lao PDR (Fooden 1996; Nadler *et al.* 2003). Our earliest understanding of the natural history of the doucs was derived mainly from studies involving the red-shanked douc (see Jablonski 1998) and feeding behavior was a widely studied aspect through captive, wild, and laboratory research. Kavanagh (1972) documented the first record of food sharing behavior in monkeys in a family group of red-shanked doucs at the San Diego Zoo in which an individual voluntarily broke off a part of a leafy branch and handed it to a neighboring individual who then fed on it (four cases). Lab analysis of stomach contents of five red-shanked doucs by Pham Nhat (1994) revealed a dominance of fruit items and the plant family Moraceae was the most represented with nine species. In the wild, Lippold (1998) described the diet of red-shanked douc as being predominated by young leaves. It was not until the turn of the century that researchers began studying the ecology of the other two species of doucs; and the black-shanked douc is the focus of this chapter.

Our current understanding of the feeding ecology of black-shanked doucs in the wild comes mainly from three extensive PhD dissertations; one studying the populations in Nui Chua and Phuoc Binh national parks in Vietnam (Hoang Minh Duc 2007), another in Seima Biodiversity Conservation Area (SBCA) in Cambodia (Rawson 2009), and one most recently in Cat Tien National Park in Vietnam (O'Brien 2014). Each of these studies examined various aspects of the feeding ecology of black-shanked doucs, from the degree of dietary flexibility in populations inhabiting different habitats (Hoang Minh Duc 2007), the seasonal

variation in diet (Rawson 2009), to the influence of nutritional content of food items (particularly leaves) on selection (O'Brien 2014). In my current study, I attempt to contribute to a better understanding of the dietary diversity in terms of food plants of wild populations of black-shanked doucs across four sites in Vietnam (Nui Chua National Park, Cat Tien National Park, Ta Kou Nature Reserve, and Hon Heo Mountain; see **Chapter 2. Study Subjects, Sites, and Sample Collection**) using DNA metabarcoding of fecal samples. Their dietary profiles are then evaluated in relation to (1) feeding observations from the field, (2) floristic diversity within their habitats, and (3) interpopulation differences.

5.2. Previous Findings

5.2.1. Feeding Observations from the Field

Over seven months of field observations during the dry and rainy seasons (February-August 2005) in Nui Chua and Phuoc Binh national parks, Hoang Minh Duc (2007) recorded 140 taxa (74 genera and 37 families) of food plants for black-shanked doucs and identified approximately half (77) to species (Table 5.1). The top families in terms of number of species consumed were Moraceae (13 species), Euphorbiaceae (10), Anacardiaceae (9), Guttiferae (9), Myrtaceae (9), and Rutaceae (9). Over seven months (December 2003-June 2004) in SBCA (the total field period was 20 months, but the author noted that feeding observations were mainly from seven consecutive months due to insufficient number of feeding observations in early months of the study; p.157), Rawson (2009) recorded 35 taxa (30 genera and 21 families) of food plants and identified 19 to species (Table 5.1). The top families in terms of number of species consumed were Fabaceae (9 species), Meliaceae (3), Combretaceae (2), Dipterocarpaceae (2), Lythraceae (2), and Moraceae (2). In Cat Tien National Park, O'Brien (2014) presented a detailed analysis of the chemical composition and nutritional content of leaves consumed and not consumed so as to evaluate their influence toward food selection of black-shanked doucs. Since his focus was not to document the total

number of food plants consumed, only three species (*Afzelia xylocarpa*, *Alphonsea gaudichaudiana*, and *Tetrameles nudiflora*) belonging to three families (Fabaceae, Annonaceae, and Tetramelaceae respectively) were identified. In total, 92 food plant taxa were identified for black-shanked doucs in Vietnam and Cambodia (Hoang Minh Duc 2007; Rawson 2009; O'Brien 2014).

Table 5.1. Food plants of black-shanked doucs based on field observations in Vietnam and Cambodia

	Hoang Minh Duc 2007 (Nui Chua & Phuoc Binh, Vietnam)	**Rawson 2009 (SBCA, Cambodia)**
Family	Species	
Anacardiaceae	*Buchanaria* cf. *siamensis*	
		Dracontomelon sp.
	Lannea coromandelica *Mangifera* sp.1 *Mangifera* sp.2 *Pentaspadon* aff. *poilanei* *Semecarpus perniciosa* *Spondias pinnata* *Swintonia* aff. *minuta* *Swintonia floribunda*	
Annonaceae		*Melodorum* sp.
	Polyalthia cf. *cerasoides* *Uvaria fauveliana*	
Apocynaceae	*Alstonia angustifolia* *Alstonia macrophylla* *Melodinus myrtifolius*	
Aquifoliaceae		*Ilex umbellata*
Araliaceae	*Schefflera canaensis* *Schefflera octophylla* *Schefflera* sp.1 *Schefflera* sp.2	
Avagaceae	*Dracaena cochinchinensis*	
Bignoniaceae	*Stereospermum* sp.	
Burseraceae	*Bursera* cf. *serrata* *Canarium subulatum*	
Combretaceae		*Anogeissus acuminata* *Terminalia* sp.
Dilleniaceae	*Dillenia blanchardii*	
		Dillenia sp.
Dipterocarpaceae		*Dipterocarpus alatus*
	Hopea ferrea	
	Hopea odorata	*Hopea odorata*
Ebenaceae	*Diospyros* sp.1 *Diospyros* sp.2	
Euphorbiaceae	*Antidesma* sp.1 *Antidesma* sp.2 *Antidesma* sp.3	

	Aporusa sp. *Bischofia javanica* *Breynia* sp. *Drypetes* sp. *Mallotus* sp. sp.15 sp.17	
Fabaceae		*Acacia concinna*
	Afzelia xylocarpa	*Afzelia xylocarpa*
		Albizia myriophylla *Bauhinia* sp.
	Dialium cochinchinensis	
		Millettia sp. *Peltophorum* cf. *dasyrrhachis*
	Peltophorum pterocarpum	
	Sindora siamensis	*Sindora siamensis*
		Xylia xylocarpa sp.
Fagaceae	*Castanopsis* sp. sp.8	
Gnetaceae	*Gnetum latifolium* var. *funicculare*	
Guttiferae	*Callophyllum ceriferum* *Callophyllum* sp.1 *Callophyllum* sp.2 *Garcinia aff. harmandii* *Garcinia cf. merguensis* *Garcinia oligantha* *Garcinia schefferi* *Garcinia* sp.1 *Garcinia* sp.2	
Hypericaceae		*Cratoxylum* sp.
Irvingiaceae	*Irvingia malayana*	*Irvingia malayana*
Lamiaceae	*Vitex canescens* *Vitex* cf. *leptobotrys* *Vitex peduncularis* *Vitex pinnata* var. *ptilota* *Vitex* sp.1 *Vitex* sp.2	
		Vitex sp2.
Lauraceae	*Dehaasia* sp. sp.14	
Lecythidaceae	*Barringtonia acutangula*	
Loganiaceae	*Strychnos minor* *Strychnos* sp.1 *Strychnos* sp.2	
Lythraceae		*Lagerstroemia* cf. *calyculata* *Lagerstroemia* sp.
Meliaceae	*Aglaia euphoroides*	
		cf. *Agalia*
	Amoora gigantea	
		Dysoxylum sp. *Heynea velutina*
Mimosoideae	*Albizia corniculata* *Albizia* sp.1	

	Albizia sp.2 *Archidendron balansae* *Archidendron chevalieri* *Archidendron pellitum*	
Moraceae	*Artocarpus rigida asperulus* *Ficus altissima* *Ficus callophylla var. callophylla* *Ficus curtipes* *Ficus depressa* *Ficus cf. lamponga* *Ficus phanrangensis*	
		Ficus racemosa
	Ficus cf. *subgelderi* *Ficus superba* var. *japonica* *Ficus tinctoria* *Ficus tjakela*	
		Ficus sp.
	Ficus sp.1 *Ficus* sp.2	
Myrtaceae	*Cleistocalyx nigrans* *Syzygium formosum* var. *ternifolium* *Syzygium* cf. *grandis* *Syzygium levinei* *Syzygium pachysarcum* *Syzygium ripicola*	
		Syzygium sp.
	Syzygium sp.1 *Syzygium* sp.2 *Syzygium* sp.3	
Pandanaceae	*Pandanus cornifer*	
Papilionoideae	*Dalbergia* sp.1 *Dalbergia* sp.2 *Derris* sp. *Millettia reticulata* *Ormosia* cf. *poilanei* sp.5 sp.16	
Poaceae	*Bambusa procea*	
Podocarpaceae	*Podocarpus neriifolius*	
Rhamnaceae		*Ziziphus cambodiana*
Rhizophoraceae	*Carallia lancaeifolia* *Carallia* sp.	
Rubiaceae		*Haldina cordifolia*
	Morinda tomentosa *Naucleae* sp. *Psychotria* sp. *Tarenna asiatica*	
Rutaceae	*Atalantia* sp.1 *Atalantia* sp.2 *Clausena* sp. *Glycosmis* sp. *Murraya* sp. *Zanthoxylum rhetsa* sp.2	

	sp.11 sp.12	
Sapindaceae	*Allophylus* sp.	
		Nephelium hypoleucum
	Nephelium melliferum *Nephelium* sp. *Xerospermum noronhianum*	
Sapotaceae	*Mimusops* sp. *Pouteria* cf. *obovata* sp.13	
Staphylaeaceae		*Turpinia* sp.
Sterculiaceae		*Pterocymbium* sp.
	Sterculia lissophylla *Sterculia* sp.	
Symplocaceae	*Symplocos adenophylla* var. *touranensis* *Symplocos* aff. *guillauminii* *Symplocos* sp.1 *Symplocos* sp.2	
Tetramelaceae		*Tetrameles nudiflora*
Theaceae	*Camellia* sp.1 *Camellia* sp.2 *Gordonia axillaris* sp.1	
Vitaceae	*Cissus* sp. *Tetrastigma* sp.	

There is very little overlap in the food plants identified between Hoang Minh Duc (2007) and Rawson (2009). A total of 24 families recorded by Hoang Minh Duc (2007) were not recorded in Rawson (2009). Conversely, there were seven families in Rawson (2009) that were not recorded in Hoang Minh Duc (2007). Only four species overlapped in both studies as food plants of the doucs: *Hopea odorata* (Dipterocarpaceae), *Afzelia xylocarpa* (Fabaceae), *Sindora siamensis* (Fabaceae), and *Irvingia malayana* (Irvingiaceae). Several reasons may explain this dissimilarity. The black-shanked doucs in SBCA were believed to be highly selective in their diet, with 44% of all feeding records (feeding from a total of 190 trees) coming from only five species (Rawson 2009), which may explain the low number of total food plant taxa recorded (35) as compared to that from the doucs in Nui Chua and Phuoc Binh (140 taxa). In addition, there were large numbers of feeding records taken from unidentified plants (51 feeding trees of up to 46 species; Rawson 2009) in SBCA while only up to 12 different plant species could not be positively identified in Nui Chua and Phuoc Binh

(Hoang Minh Duc 2007). Moreover, the botanical compositions of these habitats are also different. Nui Chua is located within the driest part of Vietnam with evergreen and deciduous forests and scrubland, Phuoc Binh has a wet and humid climate, characterized by evergreen and broadleaf forests and grasslands, while SBCA has wet and dry seasons with semi-evergreen and deciduous dipterocarp forests (Hoang Minh Duc *et al.* 2009; Rawson 2009).

The first research aim of this chapter is to describe the diet of the black-shanked doucs in terms of plant taxa using DNA metabarcoding on fecal samples collected in Nui Chua National Park, Cat Tien National Park, Ta Kou Nature Reserve, and Hon Heo Mountain. Results based on this approach are then compared with those documented by Hoang Minh Duc (2007) and Rawson (2009) to give a preliminary assessment of the value and suitability of this DNA-based approach in relation to field observation method. Given that Moraceae and Fabaceae were the top families based on number of taxa consumed in the diet documented by field observations (15 taxa and 11 taxa respectively; see Table 5.1), I expect Moraceae and Fabaceae to be among the top families in terms of the number of taxa recorded from the fecal samples collected from my four study sites combined (Hypothesis 1).

5.2.2. Floristic Diversity in Ta Kou and Cat Tien

Black-shanked doucs have been considered selective feeders in terms of the number of plant species selected in relation to the total flora species available within their habitats; they were observed to feed on only 10.6% and 7.6% of total flora species in Nui Chua and Phuoc Binh national parks respectively (Hoang Minh Duc 2007). In SBCA, the black-shanked doucs also demonstrated a highly specialized diet with 44% of all feeding records coming from only five species (see above **5.2.1. Feeding Observations from the Field**; Rawson 2009). The second aim of this chapter is to examine the dietary profiles of the douc populations in relation to the floristic diversity within their habitats, specifically in Ta Kou Nature Reserve and Cat Tien National Park.

Ta Kou Nature Reserve is a 119 km^2 forest of mainly three vegetation types: tropical evergreen seasonal sub-mountain forest, tropical semi-deciduous sub-mountain forest and tropical drought-deciduous broad-leaved lowland forest (Luu Hong Truong 2000, 2001). A total of 595 plant taxa in 365 genera and 117 families were recorded from Ta Kou Nature Reserve. The top 10 families in terms of the highest number of taxa recorded are: Fabaceae (47 spp.), Rubiaceae (33), Malvaceae (31), Orchidaceae (20), Moraceae (19), Phyllanthaceae (16), Poaceae (16), Annonaceae (15), Rutaceae (14), Asclepediaceae (13), Euphorbiaceae (13), and Vitaceae (13).

The vegetation types in the much larger Cat Tien National Park (740 km^2) are relatively more diverse, consisting of tropical evergreen forest, semi-evergreen forest, bamboo forest, mixed tree and bamboo forest, scrub, grassland, and wetlands and lakes (Monastyrskii 2000). Here, a total of 1607 plant taxa in 697 genera and 167 families were recorded (Cat Tien National Park 2012) and the forest is dominated by *Lagerstroemia* (Lythraceae) and *Dipterocarpus* (Dipterocarpaceae) (Blanc *et al.* 2000). The top 10 families in terms of the highest number of taxa recorded were: Fabaceae (133 spp.), Orchidaceae (118), Rubiaceae (95), Poaceae (73), Malvaceae (57), Phyllanthaceae (51), Euphorbiaceae (44), Cyperaceae (43), Lauraceae (42), and Verbenaceae (41).

Nui Chua National Park covers an area of 225 km^2 of forest and is characterized by five main vegetation zones: thorny scrubland, dry thorny scrub and woodland, dry deciduous forest and tropical savannah woodland, sclerophyll evergreen forest, and sub-montane evergreen forest (Sub-FIPI 1996). A total of 1,265 plant species belonging to 596 genera from 147 families were recorded from the national park (see Hoang Minh Duc 2007; unpublished report from Sub-FIPI 2002) but the flora list was not available.

Lastly, Hon Heo Mountain covers an area of approximately 150 km^2 with mainly tropical dry forest (Ha Thang Long & Nadler 2007). Because of a lack of research on the

biodiversity of Hon Heo Mountain, which is an unprotected area, little is known about its flora diversity. Therefore, I focus only on investigating dietary selectivity of the doucs in relation to the floristic diversity within Ta Kou Nature Reserve and Cat Tien National Park. Given in both Ta Kou and Cat Tien that Fabaceae was the dominant family based on number of taxa, I expect Fabaceae to be among the top families in terms of the number of taxa recovered from the fecal samples from Ta Kou (Hypothesis 2a) and Cat Tien (Hypothesis 2b). Finally, I aim to investigate whether there are interpopulation differences in the dietary profiles between black-shanked doucs from the four study sites, and I expect to detect a difference in their dietary profiles (Hypothesis 3).

5.3. Diet Metabarcoding Results

5.3.1. Number of Plant Identifications

A total of 37 black-shanked douc fecal samples were processed for diet metabarcoding. After applying the bioinformatics filtering criteria (see **Chapter 4.4. on Data Analysis**), 36 fecal samples were retained (Table 5.2). Only one sample (L81) from Cat Tien National Park was rejected because its Renkonen similarity index (RE) was 0.039 which was lower than that of the negative control (0.131), indicating that the amplified sequences between the replicates of L81 were highly dissimilar, which were likely due to unreliable amplifications and/or contamination. In total, 110 unique sequences (or Molecular Operational Taxonomic Units, MOTUs) with ≥90% best identity score to any taxonomic assignment were recorded (Appendix C). Among these, only six MOTUs were found across all four sites, and they were identified as Moraceae, Lamiaceae, Annonaceae, Lauraceae, lamiids (clade), and Sapotaceae using the global database. The average number of unique sequences detected in each fecal sample was 13.9 (minimum of three and maximum of 21) and most samples (88.9%) had more than 10 sequences detected (Figure 5.1). When examining the results by the sites, the average number of unique sequences detected in each

fecal sample collected from Hon Heo, Nui Chua, Cat Tien, and Ta Kou was 16 ± 3.7, 14.4 ± 4.6, 14.3 ± 3.5, and 13.3 ± 3.7 respectively (Table 5.2, Figure 5.2), which were not significantly different between the sites (one-way ANOVA, p=0.579, Appendix D).

Table 5.2. Number of unique sequences (MOTUs) from BSD samples across the sites.

	Hon Heo	**Nui Chua**	**Cat Tien**	**Ta Kou**
# of samples	4	5	7	20
Average # of MOTUs (≥90% identity score) per sample	16.0±3.7	14.4±4.6	14.3±3.5	13.3±3.7
# of MOTUs	28	42	44	45
# of exclusive MOTUs	10	20	21	25
Total # of MOTUs	110			
# of shared MOTUs across all sites	6			

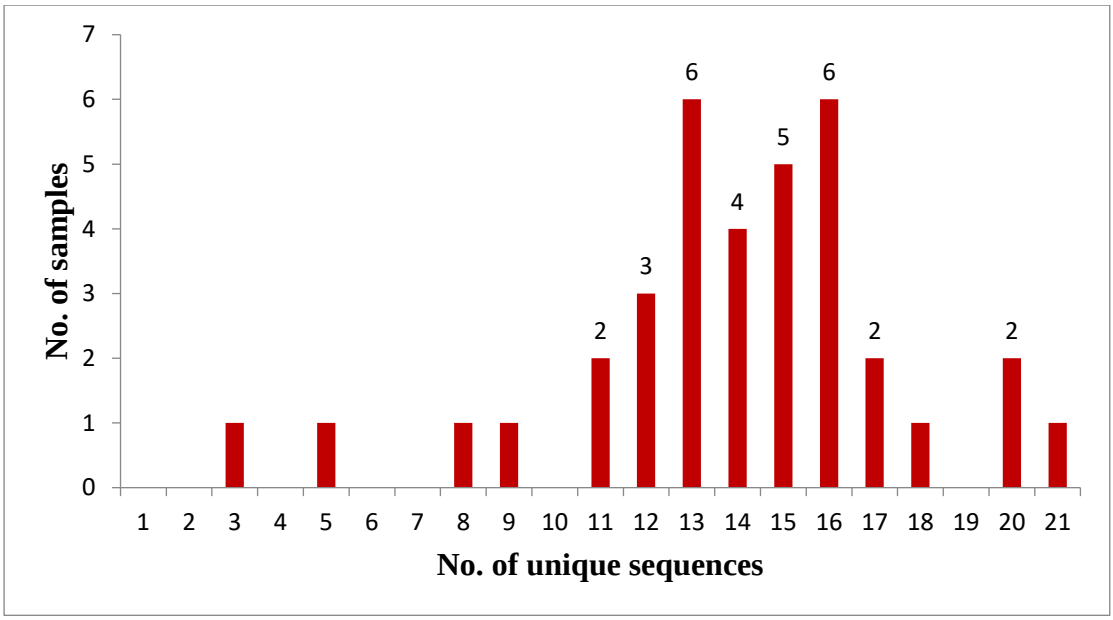


Figure 5.1. Number of unique sequences recorded in each fecal sample (BSD) from four sites (Cat Tien, Ta Kou, Nui Chua, and Hon Heo) combined. Bars with no data labels indicate that they only occurred once.

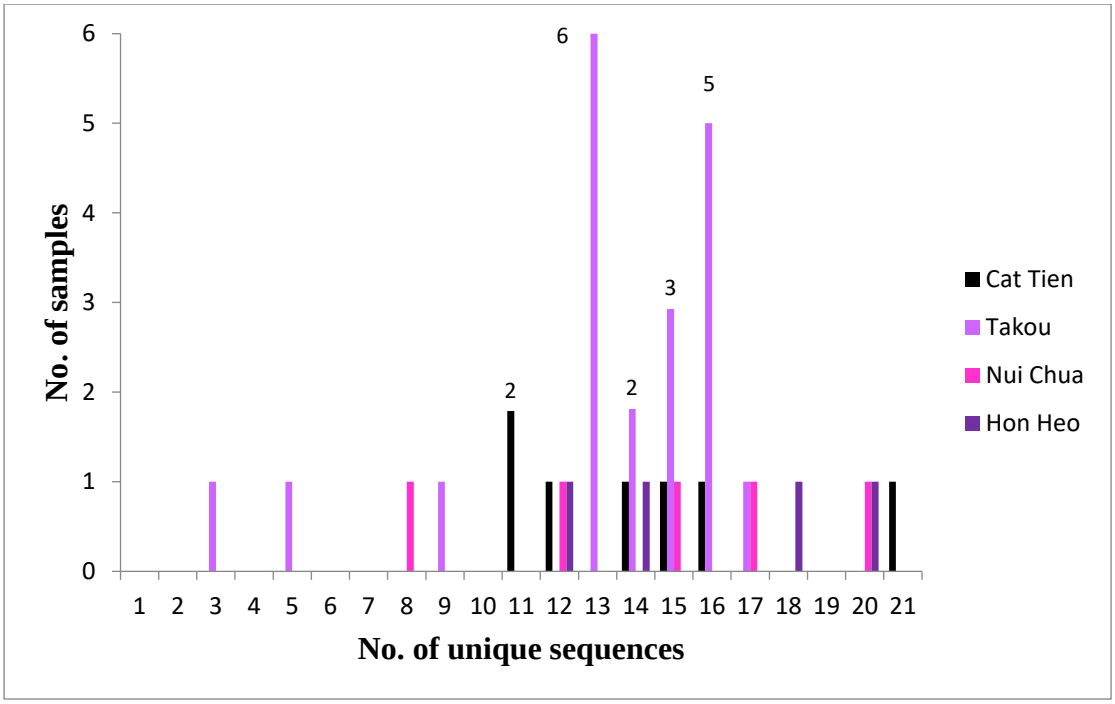


Figure 5.2. Number of unique sequences recorded in each fecal sample (BSD) from each site. Bars with no data labels indicate that they only occurred once.

5.3.2. Taxonomic Levels of Plant Identifications

Different reference databases were built for each of the four sites. For Ta Kou Nature Reserve, three databases were built using GenBank chloroplast trnL sequences of 1) angiosperms (global database); 2) plant taxa within the genera known to occur in Ta Kou Nature Reserve (local genus database), and; 3) plant taxa known to occur in Ta Kou Nature Reserve (local species database) (following the method of Quéméré *et al.* 2013). Table 5.3 shows the taxonomic levels of plant identifications using the three different databases for Ta Kou. With the global database, 21 of 45 (46.7%) sequences were identified to at least family level with ≥95% best identity score (Table 5.3). The majority of the sequences (28.9%) were identified to family level. With the local genus database, 24 of 45 (53.3%) sequences were identified to at least family level, with equal proportion of sequences (24.4%) with either no identification or identification to the genus level. Note that 93 of 365 (25.5%) genera known to occur in Ta Kou did not have sequences in GenBank. Finally, using the local species database, only 10 of 45 (22.2%) sequences were identified to at least family level. More than

half of the sequences (57.8%) did not have an identification. Note that 441 of 595 (74.1%) taxa known to occur in Ta Kou did not have sequences in GenBank.

Table 5.3. Number of sequences at each level of identification using global and local databases (Ta Kou Nature Reserve).

Level of identification	**Global database**	**Local (Genus) database**	**Local (Species) database**	**Final identification**
NA	4	11 (24.4%)	26 (57.8%)	17 (37.8%)
<95%	7	7	7	-
Higher than family	6	1	1	-
Family	13 (28.9%)	7	1	7
Subfamily/Tribe/Subtribe	2	5	0	5
Genus	5	11 (24.4%)	2	9
Species	1	1	7	7
Incorrect identification	7	2	1	-
Total	**45**			

NA: sequences that did not have an identification; <95%: sequences given an identification but with <95% identity score; Higher than family: sequences given an identification which is higher than family level, such as order or clade; Incorrect identification: sequences given an identification with ≥95% to a genus or ≥98% to a species that is not known to occur within Ta Kou, or to a species with 95% to <98% identity score (modified from de Barba *et al.* 2014).

For Cat Tien National Park, two databases were built using trnL sequences of 1) angiosperms (global database), and; 2) plant taxa within the genera known to occur in Cat Tien National Park (local genus database). A local species database was not constructed because a reliable flora checklist for Cat Tien National Park was not available (pers. comm. Dr. Luu Hong Truong). Using the global database, 31 of 44 (70.5%) sequences were identified to at least family level with ≥95% best identity score (Table 5.4). A majority of the sequences (25.0%) were identified to family level. Similar to the results from global database, 32 of 44 (72.7%) sequences were identified to at least family level using local genus database. The majority of sequences (40.9%) were identified to genus level. Note that 194 of 697 (27.8%) genera known to occur in Cat Tien did not have sequences in GenBank.

Table 5.4. Number of sequences at each level of identification using global and local databases (Cat Tien National Park).

Level of identification	**Global database**	**Local (Genus) database**	**Final identification**
NA	3	4	9
<95%	1	2	-
Higher than family	7	5	-
Family	11 (25.0%)	8	8
Subfamily/Tribe/Subtribe	9	4	7
Genus	10	18 (40.9%)	18 (40.9%)
Species	1	2	2
Incorrect identification	2	1	-
Total	**44**		

NA: sequences that did not have an identification; <95%: sequences given an identification but with <95% identity score; Higher than family: sequences given an identification which is higher than family level, such as order or clade; Incorrect identification: sequences given an identification with ≥95% to a genus or ≥98% to a species that is not known to occur within Cat Tien, or to a species with 95% to <98% identity score (modified from de Barba *et al.* 2014).

For both Nui Chua National Park and Hon Heo Mountain, only a global database using trnL sequences of angiosperms was built because a flora checklist of the two areas was not available. Using the global database, approximately 70% of the sequences from Nui Chua (29 of 42) and from Hon Heo (20 of 28) were identified to at least family level (Table 5.5), and about one-third of the sequences were identified to family level.

Table 5.5. Number of sequences at each level of identification using global database (Nui Chua National Park and Hon Heo Mountain).

Level of identification	**Nui Chua**		**Hon Heo**	
	Global database	**Final**	**Global database**	**Final**
NA	0	13	1	8
<95%	5	-	4	-
Higher than family	3	-	2	-
Family	14 (33.3%)	14 (33.3%)	9 (32.1%)	9 (32.1%)
Subfamily/Tribe/Subtribe	5	5	6	6
Genus	7	7	2	2
Species	3	3	3	3
Incorrect identification	5	-	1	-
Total	**42**		**28**	

NA: sequences that did not have an identification; <95%: sequences given an identification but with <95% identity score; Higher than family: sequences given an identification which is higher than family level, such as order or clade; Incorrect identification: sequences given an identification with ≥95% to a genus or ≥98% to a species that is not known to occur within Nui Chua or Hon Heo respectively, or to a species with 95% to <98% identity score (modified from de Barba *et al.* 2014).

5.3.3. Plant Identifications for Samples

5.3.3.1. From Ta Kou Nature Reserve

A family/genus name was assigned if the sequence identity was ≥95% to the database and species name if the identity was ≥98% (following the method of de Barba *et al.* 2014). Table 5.6a details the identifications of the 45 sequences from the black-shanked douc samples collected from Ta Kou Nature Reserve using the global, local genus, and local species databases. Identifications varied depending on which database was used as the reference. The finalized list of plant identifications is presented in Table 5.6b after consolidating the different taxonomic levels of identifications. For instance, sequence no. 12 was identified only to Anacardiaceae (family level) when using the global or local genus database, both at 100% identity matches, but the identification was refined to *Bouea oppositifolia*/Anacardiaceae (species level) at 100% identity match when using the local species database. Therefore, the final identification for sequence no. 12 was *Bouea oppositifolia*/Anacardiaceae.

The identifications were also manually checked for inaccurate identifications, such as to species or genera not known to be found in Ta Kou or Vietnam. For example, sequence no. 5 was assigned to *Petitia domingensis*/Lamiaceae (species level) at 98% identity match, but when queried using the local genus or local species database, no identification was given. Upon inspection, I determined the species level identification as an incorrect identification as *Petitia domingensis* is only found in Florida and the West Indies and is not known to occur in Vietnam. Therefore, sequence no. 5 remained unidentified. Similarly, sequence no. 19 was identified as *Calliandra umbellifera*/Fabaceae (species level) at 100% identity match using the global database, as *Derris elliptica*/Fabaceae (species level) at 98% identity match using the local genus database, and was not identified using the local species database. The genus *Calliandra* is native to the Americas (de Souza *et al.* 2013) and so *C. umbellifera* was

Table 5.6a. Plant identifications for the black-shanked douc samples from Ta Kou Nature Reserve (total samples = 20) based on global and local databases. Assignments are regardless of identity score.

Sequence no.	Taxon			No. of samples
	Global database	Local (Genus) database	Local (Species) database	
1	**Annonaceae**/*Artabotrys* (1.00)	**Annonaceae**/*Artabotrys* (1.00)	**Annonaceae**/*Desmos dinhensis* (0.94)	18
2	**Bignoniaceae** (1.00)	**Bignoniaceae**/*Stereospermum* (0.98)	NA	18
3^	**Fagaceae** (1.00)	**Fagaceae** (1.00)	NA	18
4	**Moraceae** (1.00)	**Moraceae** (1.00)	**Moraceae** (1.00)	18
5	**Lamiaceae**/*Petitia domingensis* (0.98)	NA	NA	18
6	**Menispermaceae**/Menispermoideae (1.00)	(Magnoliidae) (0.92)	NA	16
7^	**Capparaceae**/*Stixis* (1.00)	**Capparaceae**/*Stixis* (1.00)	NA	15
8^	**Menispermaceae** (1.00)	**Menispermaceae**/Menispermoideae (0.96)	**Menispermaceae**/*Cyclea barbata* (0.94)	15
9	**Apocynaceae**/*Parameria laevigata* (1.00)	**Apocynaceae**/*Parameria laevigata* (1.00)	**Apocynaceae**/*Parameria laevigata* (1.00)	13
10^	(Malpighiales) (0.93)	**Phyllanthaceae**/*Phyllanthus* (0.93)	NA	11
11	NA	NA	NA	11
12^	**Anacardiaceae** (1.00)	**Anacardiaceae** (1.00)	**Anacardiaceae**/*Bouea oppositifolia* (1.00)	10
13^	(Cornales) (1.00)	**Asteraceae**/Asteroideae (0.96)	(Asterids) (0.94)	10
14	**Annonaceae** (1.00)	**Annonaceae**/*Polyalthia* (1.00)	**Annonaceae**/*Polyalthia* (0.98)	9
15	**Fabaceae**/*Entada* (1.00)	**Fabaceae**/*Entada* (1.00)	**Fabaceae**/*Entada pursaetha* (1.00)	8
16	**Lauraceae** (1.00)	**Lauraceae** (1.00)	NA	8
17^	(Mesangiospermae) (0.94)	**Annonaceae** (0.94)	**Annonaceae** (0.91)	6
18^	**Menispermaceae** (0.92)	NA	NA	4
19^	**Fabaceae**/*Calliandra umbellifera* (1.00)	**Fabaceae**/*Derris elliptica* (0.98)	NA	3
20^	**Fabaceae**/*Erythrina* (1.00)	**Fabaceae**/*Erythrina* (1.00)	NA	3
21	**Fabaceae** (0.98)	**Fabaceae**/*Derris elliptica* (0.98)	NA	2
22^	(Pentapetalae) (0.96)	(Malpighiales) (0.91)	NA	2
23^	**Malvaceae** (0.98)	**Malvaceae** (0.98)	**Malvaceae**/*Firmiana simplex* (0.98)	2
24^	**Rhamnaceae**/*Pomaderris* (0.98)	(Pentapetalae) (0.92)	NA	2
25^	NA	NA	NA	2
26	(Cornales) (1.00)	**Asteraceae**/Asteroideae (0.96)	**Asteraceae**/Asteroideae (0.94)	2
27^	**Annonaceae** (1.00)	**Annonaceae** (1.00)	**Annonaceae**/*Cyathocalyx annamensis* (1.00)	1
28	(Lamiids) (1.00)	**Apocynaceae**/Apocynoideae (1.00)	**Apocynaceae**/*Holarrhena pubescens* (1.00)	1
29	**Araliaceae** (1.00)	**Araliaceae** (1.00)	NA	1
30^	(Petrosaviidae) (1.00)	**Arecaceae**/Caryoteae (1.00)	NA	1
31	**Moraceae**/*Broussonetia* (0.95)	**Cannabaceae**/*Trema* (0.95)	**Cannabaceae**/*Trema tomentosa* (0.95)	1
32	**Combretaceae**/*Terminalia* (1.00)	**Combretaceae**/*Terminalia* (1.00)	NA	1
33*	**Sapotaceae** (1.00)	**Ebenaceae**/*Diospyros* (0.96)	**Ebenaceae**/*Diospyros* (0.96)	1

34^	**Elaeocarpaceae** (1.00)	**Elaeocarpaceae**/*Elaeocarpus* (1.00)	NA	1
35^	**Fabaceae**/Cercideae (0.98)	**Fabaceae**/*Bauhinia* (0.98)	**Fabaceae**/*Bauhinia corymbosa* (0.98)	1
36	(Fabids) (1.00)	(Fabids) (1.00)	(Fabids) (1.00)	1
37^	**Menispermaceae** (0.94)	**Menispermaceae**/Menispermoideae (0.91)	**Menispermaceae**/*Cyclea barbata* (0.91)	1
38^	(Mesangiospermae) (0.94)	(Pentapetalae) (0.92)	(Pentapetalae) (0.90)	1
39	**Celastraceae** (1.00)	NA	NA	1
40	**Combretaceae**/*Conocarpus erectus* (0.93)	NA	NA	1
41^	**Malvaceae**/Bombacoideae (0.93)	NA	NA	1
42^	NA	NA	NA	1
43^	NA	NA	NA	1
44^	**Lamiaceae**/*Congea* (1.00)	NA	NA	1
45^	**Meliaceae**/*Melia azedarach* (0.98)	NA	NA	1

*Sequence no. 33 is the same sequence as the one identified in the positive control for *Manilkara zapota* (Sapotaceae).
^sequences only found in Ta Kou Nature Reserve.

deemed as an incorrect identification. Even though *Derris elliptica* can be found in Vietnam (Bailey & Bailey 1976), only *Derris* sp. was recorded in the flora checklist of Ta Kou Nature Reserve (Luu Hong Truong 2000, 2006). Nonetheless, Fabaceae was positively identified using the global and local genus databases and therefore, I assigned Fabaceae (98% identity match) as the final identification of sequence no. 19. The names of all identified sequences were also checked against an online taxonomic list of plants (The Plant List 2013a) in order to correct for synonyms. In this case, no synonyms were found and taxonomic names in Table 5.6b were up-to-date.

In the final list of identifications (Tables 5.3 and 5.6b), 28 of 45 (62.2%) sequences were identified to at least family level, with just under 40% of the sequences not identified. A total of 19 families were recorded from the black-shanked douc fecal samples from Ta Kou Nature Reserve, with Annonaceae, Bignoniaceae, Fagaceae, and Moraceae being the most common families, being found in 18 of 20 samples (Figure 5.3). Sixteen genera (*Artabotrys*, *Bauhinia*, *Bouea*, *Cyathocalyx*, *Diospyros*, *Elaeocarpus*, *Entada*, *Erythrina*, *Firmiana*, *Holarrhena*, *Parameria*, *Polyalthia*, *Stereospermum*, *Stixis*, *Terminalia*, and *Trema*) and seven species (*Bauhinia corymbosa*, *Bouea oppositifolia*, *Cyathocalyx annamensis*, *Entada pursaetha*, *Firmiana simplex*, *Holarrhena pubescens*, and *Parameria laevigata*) were identified. Eight families (Arecaceae, Asteraceae, Cannabaceae, Capparaceae, Celastraceae, Elaeocarpaceae, Malvaceae, and Menispermaceae), four genera (*Artabotrys*, *Erythrina*, *Stixis*, and *Trema*) and all seven species listed above are new records.

Table 5.6b. Finalized list of plant identifications (Ta Kou Nature Reserve).

Sequence no.	Taxon	No. of samples
1	**Annonaceae**/*Artabotrys* (1.00)	18
2	**Bignoniaceae**/*Stereospermum* (0.98)	18
3^	**Fagaceae** (1.00)	18
4	**Moraceae** (1.00)	18
5	NA	18
6	**Menispermaceae**/Menispermoideae (1.00)	16
7^	**Capparaceae**/*Stixis* (1.00)	15
8^	**Menispermaceae**/Menispermoideae (0.96)	15

9	**Apocynaceae**/*Parameria laevigata* (1.00)	13
10^	NA	11
11	NA	11
12^	**Anacardiaceae**/*Bouea oppositifolia* (1.00)	10
13^	**Asteraceae**/Asteroideae (0.96)	10
14	**Annonaceae**/*Polyalthia* (1.00)	9
15	**Fabaceae**/*Entada pursaetha* (1.00)	8
16	**Lauraceae** (1.00)	8
17^	NA	6
18^	NA	4
19^	**Fabaceae** (0.98)	3
20^	**Fabaceae**/*Erythrina* (1.00)	3
21	**Fabaceae** (0.98)	2
22^	NA	2
23^	**Malvaceae**/*Firmiana simplex* (0.98)	2
24^	NA	2
25^	NA	2
26	**Asteraceae**/Asteroideae (0.96)	2
27^	**Annonaceae**/*Cyathocalyx annamensis* (1.00)	1
28	**Apocynaceae**/*Holarrhena pubescens* (1.00)	1
29	**Araliaceae** (1.00)	1
30^	**Arecaceae**/Caryoteae (1.00)	1
31	**Cannabaceae**/*Trema* (0.95)	1
32	**Combretaceae**/*Terminalia* (1.00)	1
33*	**Ebenaceae**/*Diospyros* (0.96)	1
34^	**Elaeocarpaceae**/*Elaeocarpus* (1.00)	1
35^	**Fabaceae**/*Bauhinia corymbosa* (0.98)	1
36	NA	1
37^	NA	1
38^	NA	1
39	**Celastraceae** (1.00)	1
40	NA	1
41^	NA	1
42^	NA	1
43^	NA	1
44^	NA	1
45^	NA	1

*Sequence no. 33 is the same sequence as the one identified in the positive control for *Manilkara zapota* (Sapotaceae). ^sequences only found in Ta Kou Nature Reserve.

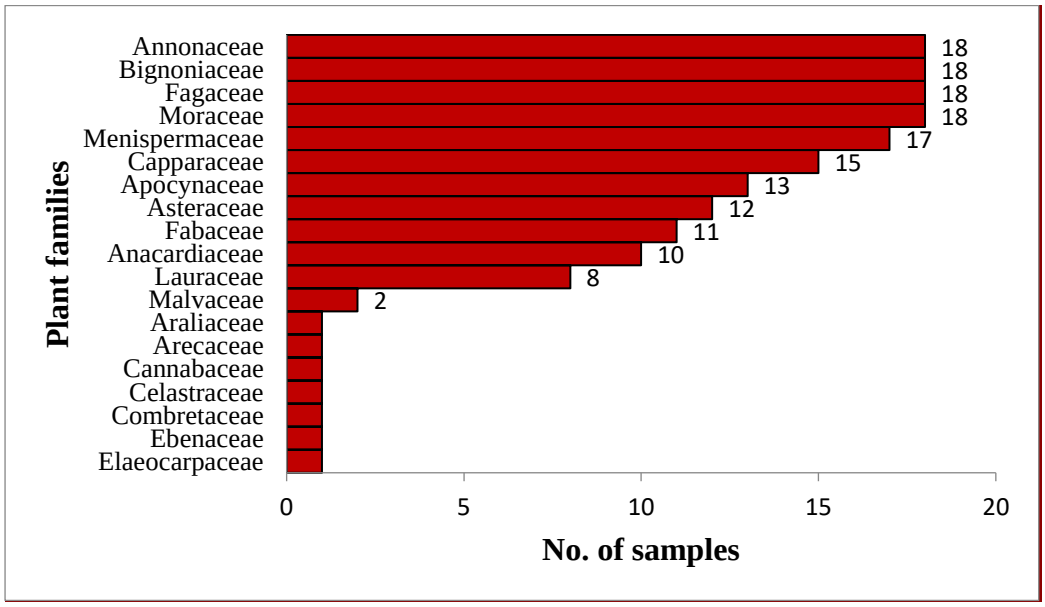


Figure 5.3. Frequency of occurrence of each plant family in the fecal samples collected from Ta Kou Nature Reserve. X-axis indicates the number of fecal samples that contained each of the plant families. Families with no data labels next to the bars (e.g. Elaeocarpaceae) indicate that they only occurred once in the samples. Annonaceae, Bignoniaceae, Fagaceae, and Moraceae were the most common families, being found in 18 of 20 samples.

5.3.3.2. From Cat Tien National Park

Table 5.7a details the identifications of the 44 sequences for the black-shanked douc samples collected in Cat Tien National Park using the global and local genus databases. The finalized list of plant identifications is presented in Table 5.7b after consolidating the different taxonomic levels of identifications. For instance, sequence no. 3 was identified only to Pentapetalae (clade level) at 100% identity match when using the global database, but the identification was refined to *Olea*/Oleaceae (family level) at 100% identity match when using the local genus database. Therefore, the final identification for sequence no. 3 was *Olea*/Oleaceae.

The identifications were also manually checked for inaccurate identifications, such as to species or genera not known to be found in Cat Tien or Vietnam. Sequence no. 41 was assigned to *Garuga*/Burseraceae (genus level) at 100% identity match using the global database, but when queried using the local genus database, it was identified to Burseraceae at a lower identity match (98%). Even though *Garuga* can be found in Vietnam, this genus was not recorded in the flora checklist of Cat Tien National Park (Cat Tien National Park 2012). Nonetheless, Burseraceae was positively identified using both databases and therefore, I assigned Burseraceae (98-100% identity match) as the final identification of sequence no. 41. In the final list of identifications (Tables 5.4 and 5.7b), 35 of 44 (79.5%) sequences were identified to at least family level, with just one-fifth of the sequences not identified.

Table 5.7a. Plant identifications for the black-shanked douc samples from Cat Tien National Park (total samples = 7) based on global and local databases. Assignments are regardless of identity score.

Sequence no.	Taxon		No. of samples
	Global database	Local (Genus) database	
1^	**Anacardiaceae**/*Buchanania* (1.00)	**Anacardiaceae**/*Buchanania* (1.00)	7
2^	**Simaroubaceae**/*Ailanthus* (1.00)	**Simaroubaceae**/*Ailanthus* (1.00)	7
3	(Pentapetalae) (1.00)	**Oleaceae**/*Olea* (1.00)	6
4	**Annonaceae** (1.00)	**Annonaceae** (1.00)	5
5	**Phyllanthaceae**/Pseudolachnostylidinae (1.00)	**Phyllanthaceae**/Pseudolachnostylidinae (1.00)	5
6	**Celastraceae** (1.00)	**Celastraceae** (1.00)	5
7	**Sapindaceae** (1.00)	**Sapindaceae** (1.00)	5
8	**Moraceae** (1.00)	**Moraceae** (1.00)	4
9	(Lamiids) (1.00)	**Apocynaceae**/Apocynoideae (1.00)	3
10	**Annonaceae**/*Artabotrys* (1.00)	**Annonaceae**/*Artabotrys* (1.00)	3
11	**Fabaceae**/Mimosoideae (1.00)	**Fabaceae**/*Acacia* (1.00)	3
12	**Fabaceae**/*Entada* (1.00)	**Fabaceae**/*Entada* (1.00)	3
13	(Fabids) (1.00)	(Fabids) (1.00)	3
14^	NA	NA	3
15^	**Dipterocarpaceae**/Dipterocarpoideae (1.00)	**Dipterocarpaceae**/Dipterocarpoideae (1.00)	3
16	**Convolvulaceae**/*Erycibe* (1.00)	**Convolvulaceae** (0.95)	2
17	(Malpighiales) (1.00)	(Malpighiales) (1.00)	2
18^	**Olacaceae**/*Olax acuminata* (1.00)	**Olacaceae**/*Olax acuminata* (1.00)	2
19^	(Sapindales) (0.98)	(Sapindales) (0.98)	2
20^	**Euphorbiaceae**/Acalypheae (1.00)	**Euphorbiaceae**/*Mallotus* (1.00)	2
21^	**Lecythidaceae** (1.00)	**Lecythidaceae**/*Careya arborea* (1.00)	2
22	**Lauraceae** (1.00)	**Lauraceae** (1.00)	1
23*	**Sapotaceae** (1.00)	**Sapotaceae**/*Chrysophyllum* (1.00)	1
24	**Bignoniaceae** (1.00)	**Bignoniaceae**/*Stereospermum* (0.98)	1
25	**Menispermaceae**/Menispermoideae (1.00)	(Magnoliidae) (0.92)	1
26	NA	NA	1
27	**Combretaceae**/*Conocarpus erectus* (0.93)	NA	1
28	**Araliaceae** (1.00)	**Araliaceae** (1.00)	1
29	**Ebenaceae** (1.00)	**Ebenaceae**/*Diospyros* (1.00)	1
30	(Pentapetalae) (1.00)	(Pentapetalae) (1.00)	1
31	**Loganiaceae**/*Strychnos* (1.00)	**Loganiaceae**/*Strychnos* (1.00)	1

32^	**Oleaceae**/*Myxopyrum nervosum* (1.00)	**Oleaceae**/*Olea* (0.95)	1
33^	**Fabaceae**/Detarieae (1.00)	**Fabaceae**/*Afzelia* (1.00)	1
34^	**Anacardiaceae**/*Buchanania* (0.96)	**Anacardiaceae**/*Buchanania* (0.96)	1
35^	**Apocynaceae**/Asclepiadeae (1.00)	(Mesangiospermae) (0.98)	1
36^	**Oleaceae**/*Jasminum* (0.98)	**Oleaceae**/*Jasminum* (0.98)	1
37^	**Lamiaceae**/*Congea* (0.96)	**Lamiaceae**/*Congea* (0.96)	1
38^	(Sapindales) (0.96)	**Sapindaceae** (0.94)	1
39^	NA	NA	1
40^	**Bignoniaceae** (1.00)	**Bignoniaceae**/*Stereospermum* (1.00)	1
41^	**Burseraceae**/*Garuga* (1.00)	**Burseraceae** (0.98)	1
42^	**Lamiaceae**/*Premna* (1.00)	**Lamiaceae**/*Premna* (1.00)	1
43^	**Apocynaceae**/Asclepiadoideae (1.00)	**Apocynaceae**/Marsdenieae (0.97)	1
44^	**Euphorbiaceae**/Erismantheae (0.97)	**Euphorbiaceae**/*Erismanthus obliquus* (0.97)	1

*Sequence no. 23 is the same sequence as the one identified in the positive control for *Manilkara zapota* (Sapotaceae).
^sequences only found in Cat Tien National Park.

Table 5.7b. Finalized list of plant identifications (Cat Tien National Park).

Sequence no.	Taxon	No. of samples
1^	**Anacardiaceae**/*Buchanania* (1.00)	7
2^	**Simaroubaceae**/*Ailanthus* (1.00)	7
3	**Oleaceae**/*Olea* (1.00)	6
4	**Annonaceae** (1.00)	5
5	**Phyllanthaceae**/Pseudolachnostylidinae (1.00)	5
6	**Celastraceae** (1.00)	5
7	**Sapindaceae** (1.00)	5
8	**Moraceae** (1.00)	4
9	**Apocynaceae**/Apocynoideae (1.00)	3
10	**Annonaceae**/*Artabotrys* (1.00)	3
11	**Fabaceae**/*Acacia* (1.00)	3
12	**Fabaceae**/*Entada* (1.00)	3
13	NA	3
14^	NA	3
15^	**Dipterocarpaceae**/Dipterocarpoideae (1.00)	3
16	**Convolvulaceae** (0.95)	2
17	NA	2
18^	**Olacaceae**/*Olax acuminata* (1.00)	2
19^	NA	2
20^	**Euphorbiaceae**/*Mallotus* (1.00)	2
21^	**Lecythidaceae**/*Careya arborea* (1.00)	2
22	**Lauraceae** (1.00)	1
23*	**Sapotaceae**/*Chrysophyllum* (1.00)	1
24	**Bignoniaceae**/*Stereospermum* (0.98)	1
25	**Menispermaceae**/Menispermoideae (1.00)	1
26	NA	1
27	NA	1
28	**Araliaceae** (1.00)	1
29	**Ebenaceae**/*Diospyros* (1.00)	1
30	NA	1
31	**Loganiaceae**/*Strychnos* (1.00)	1
32^	**Oleaceae**/*Olea* (0.95)	1
33^	**Fabaceae**/*Afzelia* (1.00)	1
34^	**Anacardiaceae**/*Buchanania* (0.96)	1
35^	**Apocynaceae**/Asclepiadeae (1.00)	1
36^	**Oleaceae**/*Jasminum* (0.98)	1
37^	**Lamiaceae**/*Congea* (0.96)	1
38^	NA	1
39^	NA	1
40^	**Bignoniaceae**/*Stereospermum* (1.00)	1
41^	**Burseraceae** (0.98-1.00)	1
42^	**Lamiaceae**/*Premna* (1.00)	1
43^	**Apocynaceae**/Marsdenieae (0.97)	1
44^	**Euphorbiaceae**/Erismantheae (0.97)	1

*Sequence no. 23 is the same sequence as the one identified in the positive control for *Manilkara zapota* (Sapotaceae).

^sequences only found in Cat Tien National Park.

A total of 24 families were recorded from the black-shanked douc fecal samples from Cat Tien National Park, with Anacardiaceae and Simaroubaceae being the most common

families, being found in all seven samples (Figure 5.4). Seventeen genera (*Acacia*, *Afzelia*, *Ailanthus*, *Artabotrys*, *Buchanania*, *Careya*, *Chrysophyllum*, *Congea*, *Diospyros*, *Entada*, *Jasminum*, *Mallotus*, *Olax*, *Olea*, *Premna*, *Stereospermum*, and *Strychnos*) and two species (*Careya arborea* and *Olax acuminata*) were identified. Seven families (Celastraceae, Convolvulaceae, Menispermaceae, Olacaceae, Oleaceae, Phyllanthaceae, and Simaroubaceae), eight genera (*Ailanthus*, *Artabotrys*, *Chrysophyllum*, *Congea*, *Entada*, *Jasminum*, *Olea*, and *Premna*) and the two species are new records.

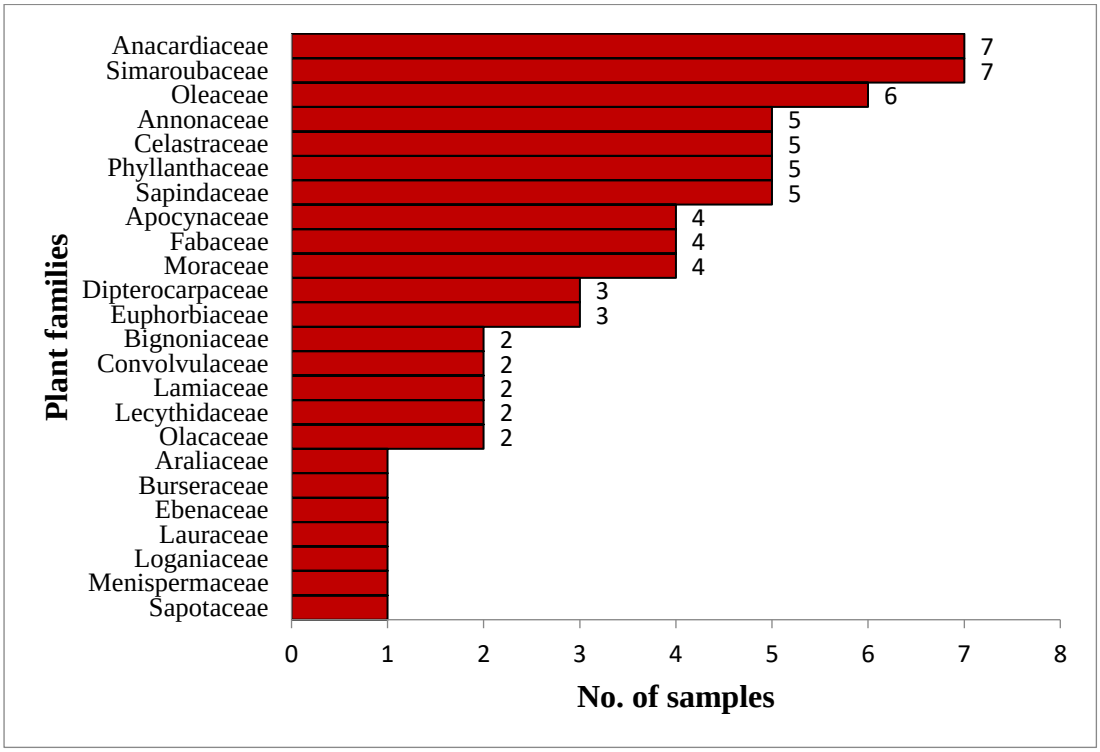


Figure 5.4. Frequency of occurrence of each plant family in the fecal samples collected from Cat Tien National Park. X-axis indicates the number of fecal samples that contained each of the plant families. Families with no data labels next to the bars (e.g. Sapotaceae) indicate that they only occurred once in the samples. Anacardiaceae and Simaroubaceae were the most common families, being found in all seven samples.

5.3.3.3. From Nui Chua National Park

Table 5.8 details the identifications of the 42 sequences from the black-shanked douc samples collected from Nui Chua National Park using the global database. The identifications were also manually checked for inaccurate identifications, such as to species or genera not known to be found in Vietnam. Sequence no. 25 was assigned to *Dilodendron bipinnatum*/Sapindaceae (species level) at 100% identity match, but this was likely an

incorrect identification as *Dilodendron bipinnatum* is found in South America. Therefore, sequence no. 25 remained unidentified. In the final list of identifications (Tables 5.5 and 5.8), 29 of 42 (69.0%) sequences were identified to at least family level, with 31% of the sequences not identified. A total of 22 families were recorded from the black-shanked douc fecal samples from Nui Chua National Park, with Phyllanthaceae being the most common family, being found in all five samples (Figure 5.5). Nine genera (*Acacia*, *Artabotrys*, *Bauhinia*, *Cordisepalum*, *Diospyros*, *Jasminum*, *Rinorea*, *Strychnos*, and *Terminalia*) and three species (*Acacia tenuifolia*, *Bauhinia championii championii*, and *Terminalia franchetii*) were identified. Seven families (Convolvulaceae, Malpighiaceae, Musaceae, Oleaceae, Phyllanthaceae, Smilacaceae, and Violaceae), four genera (*Artabotrys*, C*ordisepalum*, *Jasminum*, and *Rinorea*), and all three species are new records.

Table 5.8. Plant identifications for the black-shanked douc samples from Nui Chua National Park (total samples = 5) based on global database.

Sequence no.	Global database	Final identification (only retaining ≥95% identity score)	No. of samples
1	**Phyllanthaceae**/Bridelieae (1.00)	**Phyllanthaceae**/Bridelieae (1.00)	5
2	**Moraceae** (1.00)	**Moraceae** (1.00)	4
3	**Annonaceae** (1.00)	**Annonaceae** (1.00)	4
4	**Ebenaceae**/*Diospyros* (1.00)	**Ebenaceae**/*Diospyros* (1.00)	4
5^	**Violaceae**/*Rinorea* (1.00)	**Violaceae**/*Rinorea* (1.00)	3
6^	**Combretaceae**/*Terminalia franchetii* (1.00)	**Combretaceae**/*Terminalia franchetii* (1.00)	3
7^	**Euphorbiaceae** (0.96)	**Euphorbiaceae** (0.96)	3
8	**Lamiaceae**/*Petitia domingensis* (0.98)	NA	2
9	**Fabaceae**/*Lasiobema championii championii* (0.98)	**Fabaceae**/*Bauhinia championii championii* (0.98)	2
10	**Rhamnaceae** (0.92)	NA	2
11	(Malpighiales) (1.00)	NA	2
12	**Rubiaceae** (1.00)	**Rubiaceae** (1.00)	2
13	**Loganiaceae**/*Strychnos* (1.00)	**Loganiaceae**/*Strychnos* (1.00)	2
14	**Lauraceae** (1.00)	**Lauraceae** (1.00)	1
15	**Apocynaceae**/Apocyneae (1.00)	**Apocynaceae**/Apocyneae (1.00)	1
16*	**Sapotaceae** (1.00)	**Sapotaceae** (1.00)	1
17	**Fabaceae**/Millettieae (0.94)	NA	1
18	**Annonaceae**/*Artabotrys* (1.00)	**Annonaceae**/*Artabotrys* (1.00)	1
19	**Bignoniaceae** (1.00)	**Bignoniaceae** (1.00)	1
20	**Rubiaceae** (1.00)	**Rubiaceae** (1.00)	1
21	**Vitaceae** (1.00)	**Vitaceae** (1.00)	1
22	**Fabaceae**/*Senegalia tenuifolia* (1.00)	**Fabaceae**/*Acacia tenuifolia* (1.00)	1
23	(Cornales) (1.00)	NA	1
24	**Combretaceae**/*Terminalia* (1.00)	**Combretaceae**/*Terminalia* (1.00)	1
25	**Sapindaceae**/*Dilodendron bipinnatum* (1.00)	NA	1
26^	(Malpighiales) (0.95)	NA	1
27^	**Sapindaceae** (1.00)	**Sapindaceae** (1.00)	1
28^	**Atherospermataceae**/*Laurelia sempervirens* (0.98)	NA	1
29^	**Atherospermataceae** (0.98)	NA	1
30^	**Burseraceae** (1.00)	**Burseraceae** (1.00)	1
31^	**Connaraceae**/*Byrsocarpus coccineus* (1.00)	NA	1
32^	**Rubiaceae**/*Afrocanthium* (0.94)	NA	1
33^	**Euphorbiaceae**/Hippomaneae (0.96)	**Euphorbiaceae**/Hippomaneae (0.96)	1
34^	**Euphorbiaceae** (0.93)	NA	1

35^	**Phyllanthaceae**/Bridelieae (0.95)	**Phyllanthaceae**/Bridelieae (0.95)	1
36^	**Malpighiaceae** (1.00)	**Malpighiaceae** (1.00)	1
37^	**Capparaceae** (0.94)	NA	1
38^	**Smilacaceae** (0.98)	**Smilacaceae** (0.98)	1
39^	**Convolvulaceae**/*Cordisepalum* (1.00)	**Convolvulaceae**/*Cordisepalum* (1.00)	1
40^	**Oleaceae**/*Jasminum* (1.00)	**Oleaceae**/*Jasminum* (1.00)	1
41^	**Fabaceae**/Cassieae (1.00)	**Fabaceae**/Cassieae (1.00)	1
42^	**Musaceae** (1.00)	**Musaceae** (1.00)	1

*Sequence no. 16 is the same sequence as the one identified in the positive control for *Manilkara zapota* (Sapotaceae).
^sequences only found in Nui Chua National Park.

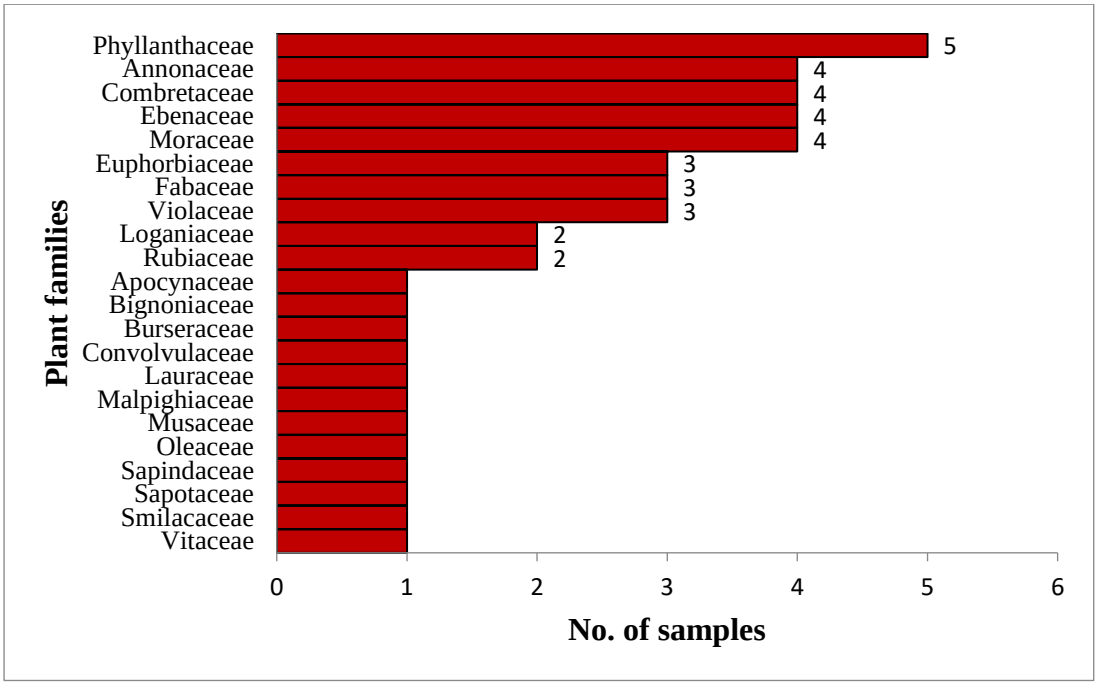


Figure 5.5. Frequency of occurrence of each plant family in the fecal samples collected from Nui Chua National Park. X-axis indicates the number of fecal samples that contained each of the plant families. Families with no data labels next to the bars (e.g. Vitaceae) indicate that they only occurred once in the samples. Phyllanthaceae was the most common family, being found in all five samples.

5.3.3.4. From Hon Heo Mountain

Table 5.9 details the identifications of the 28 sequences for the black-shanked douc samples collected in Hon Heo Mountain using the global database. The identifications were manually checked for inaccurate identifications, such as to species or genera not known to be found in Vietnam. For example, sequence no. 8 was assigned to *Petitia domingensis*/Lamiaceae (species level) at 98% identity match, but it was likely an incorrect identification as *Petitia domingensis* is only found in Florida and the West Indies and is not known to occur in Vietnam. Therefore, sequence no. 8 remained unidentified. Synonyms

were detected for identifications in the Hon Heo samples. Sequence no. 5 was identified as *Lasiobema championii* but it should be *Bauhinia championii* (see The Plant List 2013b). Similarly, sequence no. 24 was identified as *Senegalia tenuifolia*, but it should be *Acacia tenuifolia* (see The Plant List 2013c). A total of 13 families were recorded from the black-shanked douc fecal samples from Hon Heo Mountain, with Annonaceae, Fabaceae, Moraceae, and Rubiaceae being the most common families, being found in all four samples (Figure 5.6). Five genera (*Acacia*, *Bauhinia*, *Celtis*, *Erycibe* and *Prunus*) and three species (*Acacia tenuifolia*, *Bauhinia championii championii*, and *Celtis biondii*) were identified. Four families (Cannabaceae, Convolvulaceae, Phyllanthaceae, and Rosaceae), three genera (*Celtis*, *Erycibe* and *Prunus*) and all three species are new records.

Table 5.9. Plant identifications for the black-shanked douc samples from Hon Heo Mountain (total samples = 4) based on global database.

Sequence no.	Global database	Final identification (only retaining ≥95% identity score)	No. of samples
1	**Moraceae** (1.00)	**Moraceae** (1.00)	4
2	**Annonaceae** (1.00)	**Annonaceae** (1.00)	4
3	**Fabaceae**/Millettieae (0.94)	NA	4
4	NA	NA	4
5	**Fabaceae**/*Lasiobema championii championii* (0.98)	**Fabaceae**/*Bauhinia championii championii* (0.98)	4
6	**Rubiaceae** (1.00)	**Rubiaceae** (1.00)	4
7^	**Rubiaceae** (0.98)	**Rubiaceae** (0.98)	4
8	**Lamiaceae**/*Petitia domingensis* (0.98)	NA	3
9	**Lauraceae** (1.00)	**Lauraceae** (1.00)	3
10^	(Malpighiales) (0.93)	NA	3
11^	**Rubiaceae** (1.00)	**Rubiaceae** (1.00)	3
12^	**Fabaceae**/Millettieae (1.00)	**Fabaceae**/Millettieae (1.00)	3
13	(Rosales) (0.95)	NA	2
14	**Fabaceae**/Mimosoideae (1.00)	**Fabaceae**/Mimosoideae (1.00)	2
15	**Vitaceae** (1.00)	**Vitaceae** (1.00)	2
16^	**Fabaceae** (1.00)	**Fabaceae** (1.00)	2
17^	**Cannabaceae**/*Celtis biondii* (1.00)	**Cannabaceae**/*Celtis biondii* (1.00)	2
18	**Apocynaceae**/Apocyneae (1.00)	**Apocynaceae**/Apocyneae (1.00)	1
19*	**Sapotaceae** (1.00)	**Sapotaceae** (1.00)	1
20	**Phyllanthaceae**/Bridelieae (1.00)	**Phyllanthaceae**/Bridelieae (1.00)	1
21	(Pentapetalae) (1.00)	NA	1
22	**Convolvulaceae**/*Erycibe* (1.00)	**Convolvulaceae**/*Erycibe* (1.00)	1
23	**Rhamnaceae** (0.92)	NA	1
24	**Fabaceae**/*Senegalia tenuifolia* (1.00)	**Fabaceae**/*Acacia tenuifolia* (1.00)	1
25^	**Euphorbiaceae**/Acalyphoideae (0.98)	**Euphorbiaceae**/Acalyphoideae (0.98)	1
26^	**Rosaceae**/*Prunus* (1.00)	**Rosaceae**/*Prunus* (1.00)	1
27^	**Fabaceae**/Millettieae (0.96)	**Fabaceae**/Millettieae (0.96)	1
28^	(Pentapetalae) (0.92)	NA	1

*Sequence no. 19 is the same sequence as the one identified in the positive control for *Manilkara zapota* (Sapotaceae).
^sequences only found in Hon Heo Mountain.

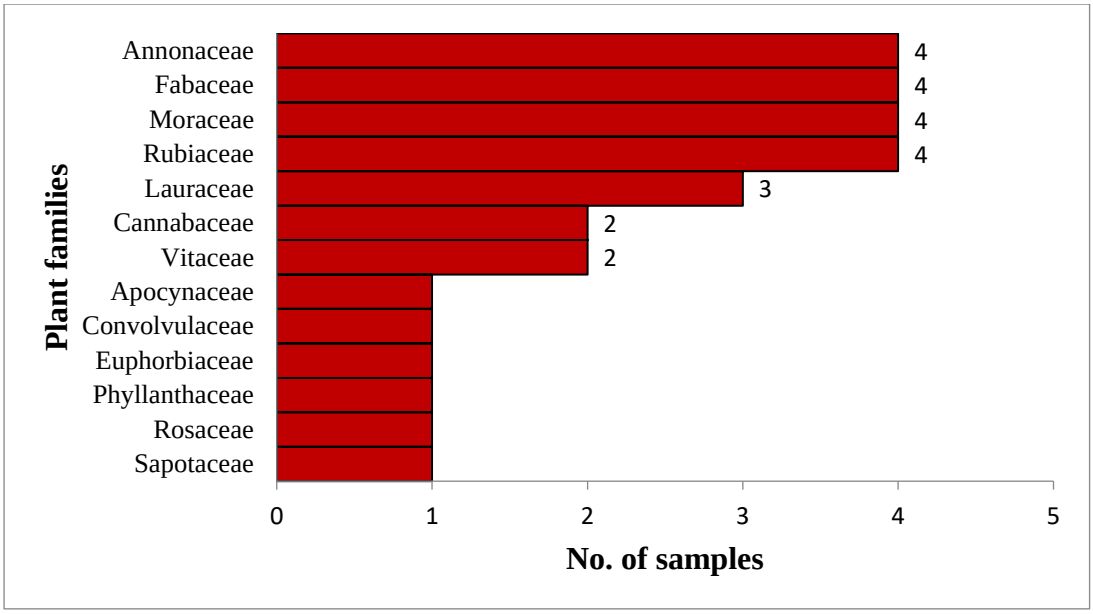


Figure 5.6. Frequency of occurrence of each plant family in the fecal samples collected from Hon Heo Mountain. X-axis indicates the number of fecal samples that had each of the plant families. Families with no data labels next to the bars (e.g. Sapotaceae) indicate that they only occurred once in the samples. Annonaceae, Fabaceae, Moraceae, and Rubiaceae were the most common families, being found in all four samples.

5.3.3.5. Summary

In sum, after consolidating the plant identifications from 36 fecal samples of the black-shanked doucs from all four sites, 40 families were recorded, with Annonaceae being the most common family (Figure 5.7). The top 10 families in terms of the highest number of fecal samples containing them were: Annonaceae (31 samples), Moraceae (30), Fabaceae (22), Apocynaceae (19), Bignoniaceae (18), Fagaceae (18), Menispermaceae (18), Anacardiaceae (17), Capparaceae (15), and Lauraceae (13). Thirty-four genera and 13 species were also recorded. A total of 18 families (Arecaceae, Asteraceae, Cannabaceae, Capparaceae, Celastraceae, Convolvulaceae, Elaeocarpaceae, Malpighiaceae, Malvaceae, Menispermaceae, Musaceae, Olacaceae, Oleaceae, Phyllanthaceae, Rosaceae, Simaroubaceae, Smilacaceae, and Violaceae), 26 genera (*Ailanthus*, *Artabotrys*, *Bouea*, *Buchanania*, *Careya*, *Celtis*, *Chrysophyllum*, *Congea*, *Cordisepalum*, *Cyathocalyx*, *Elaeocarpus*, *Entada*, *Erycibe*, *Erythrina*, *Firmiana*, *Holarrhena*, *Jasminum*, *Mallotus*, *Olax*, *Olea*, *Parameria*, *Premna*, *Prunus*, *Rinorea*, *Stixis*, and *Trema*) and all 13 species (*Acacia*

tenuifolia, *Bauhinia championii championii*, *Bauhinia corymbosa*, *Bouea oppositifolia*, *Careya arborea*, *Celtis biondii*, *Cyathocalyx annamensis*, *Entada pursaetha*, *Firmiana simplex*, *Holarrhena pubescens*, *Olax acuminata*, *Parameria laevigata*, and *Terminalia franchetii*) are new records such that there are currently 105 food plant taxa identified for black-shanked douc based on observations and metabarcoding (Appendix I).

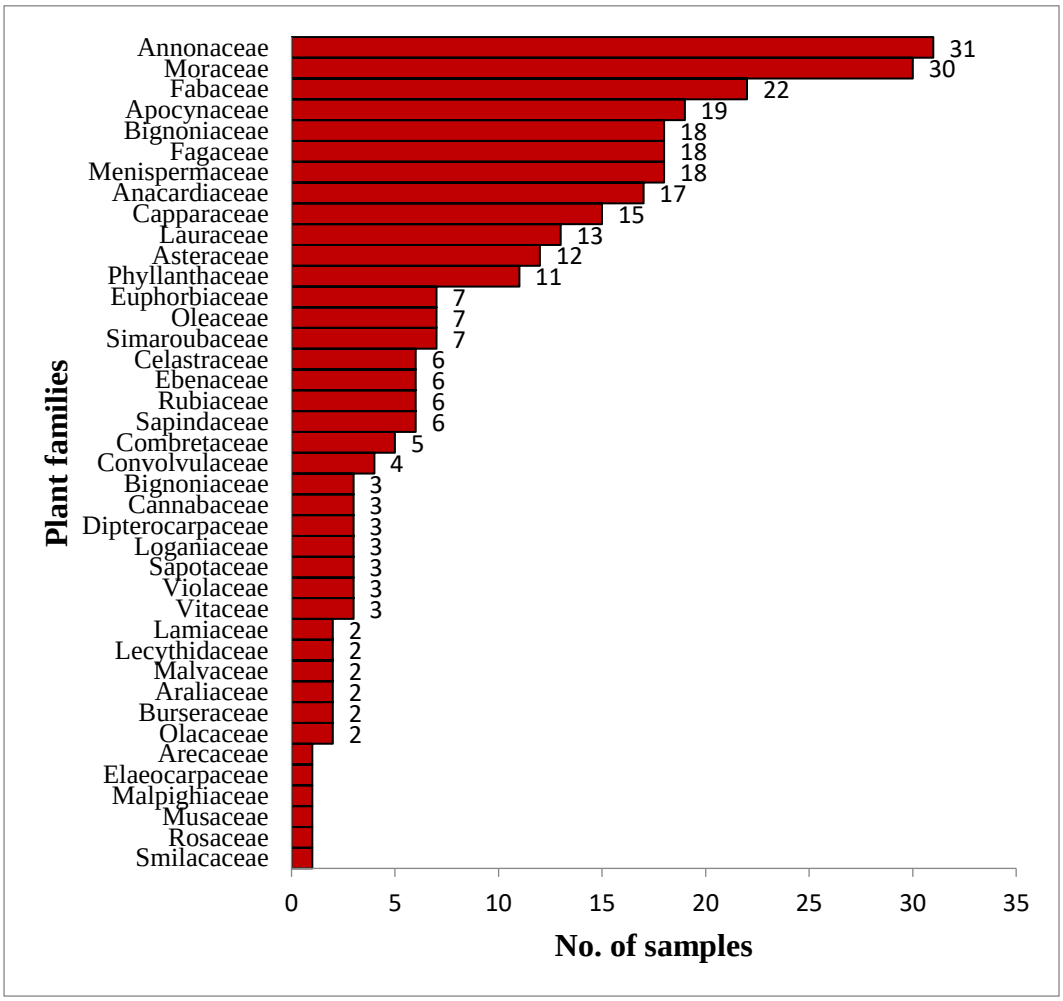


Figure 5.7. Frequency of occurrence of each plant family in the 36 fecal samples collected from all four sites. X-axis indicates the number of fecal samples that had each of the plant families. Families with no data labels next to the bars (e.g. Smilacaceae) indicate that they only occurred once in the samples. Annonaceae was the most common family, being found in 31 samples.

5.4. Discussion

Diet metabarcoding in association with next-generation sequencing has not been previously applied to fecal samples of the doucs (*Pygathrix* spp.). Here, I demonstrated a high success rate of DNA amplification, sequencing, and replicability for the diet metabarcoding of black-shanked douc fecal samples. The level and accuracy of plant identifications to each of the unique sequences detected in the black-shanked douc fecal samples varied depending on which database was used as the taxonomic reference. Among the three reference databases used for samples from Ta Kou Nature Reserve, the local genus database might be considered as performing the best with the highest proportion of sequences (24 of 45, or 53%) identified to at least family level as compared to the global and local species databases. Moreover, a majority of the sequences was identified to the genus level, which is valuable for community-level diet analysis. There were also only two incorrect identifications using the local genus database. In contrast, the local species database provided the highest number of species identifications but also resulted in almost 60% of the sequences with no identification. In this case, the use of both local databases would enhance the performance of metabarcoding in providing taxonomic identifications. For samples from Cat Tien National Park, the global and local genus databases were examined. More than 70% of the sequences were identified to at least family level using each of the databases, but the genus database worked better by identifying 40% of the sequences down to genus level. For samples from Nui Chua National Park and Hon Heo Mountain, only the global database was utilized as no flora lists of the habitats were available. Approximately 70% of the sequences from each site were also identified to at least family level, but taxonomic resolution to the genus and species levels were low (7.1%-16.7%).

5.4.1. Comparison between Metabarcoding and Feeding Observations

From a total of 36 black-shanked douc fecal samples processed, 110 unique diet sequences (or MOTUs) were recorded, demonstrating a wide dietary selection by the doucs across four study sites in Vietnam. Even though 20.5%-37.8% of the sequences from each site could not be identified, 40 plant families were still recorded in the black-shanked douc samples, with Annonaceae being the most common family in terms of the highest number of fecal samples containing them, followed by Moraceae and Fabaceae. Feeding observations (Hoang Minh Duc 2007; Rawson 2009) also revealed a dominance of Moraceae and Fabaceae based on number of taxa consumed, but Annonaceae did not contribute significantly (Hypothesis 1). There was also little overlap in the families and genera identified through previous feeding observations and diet metabarcoding in this study, and no overlap in food species; 18 families, 15 genera and 13 plant species detected through diet metabarcoding had not been recorded as food plants from field observations.

These new records included a number of liana species (five of 13 species are lianas; i.e. *Acacia tenuifolia*, *Bauhinia championii*, *B. corymbosa*, *Entada pursaetha*, and *Parameria laevigata*). Lianas are woody climbing plants which play an important role in forests dynamics such as forest regeneration, species diversity, canopy connectivity, and providing a food source for animals, particularly in tropical rainforests (Putz & Mooney 1991; see Schnitzer & Bongers 2002). In tropical forests, liana species may contribute about 25% of total species diversity (comprising tree, liana, shrub, and herb) and woody stem density within a habitat (Gentry 1991). In Xishuangbanna, southern Yunnan in China, up to 27% of the total species was lianas (Zhu 2008). Research into their ecology, however, trails behind that of tree species and other vascular plant groups (Putz & Mooney 1991) due to various reasons. Lianas are often difficult to identify in the field owing to problems collecting vegetative and/or reproductive herbarium material (Parren *et al.* 2005). It is also challenging

to distinguish young liana from a tree as many species are free standing when they begin growth (Parren *et al.* 2005). Along with these issues and taxonomic uncertainties, lianas might inadvertently be under-reported in the diet composition of primates, and the importance of lianas in forest dynamics might be downplayed (Schnitzer & Bongers 2002).

Two and a half years of field observations of the Raffles' banded langur in Singapore recorded 27 food plant species, of which six (22%) were lianas (Ang 2010). Using metabarcoding and metagenomics of fecal samples collected from the same population during the same field period, Srivathsan *et al.* 2016 recorded up to 63 plant species of which 28% were lianas. Even though diet metabarcoding is unable to identify plant parts consumed, it can complement field observations by detecting DNA sequences of plant species such as lianas which might elude the detection of field researchers. Lianas may be important food resources for primate species and may play a significant role in sustaining primate populations especially in disturbed habitats. Several studies have found that lianas become more common in disturbed forests (such as after logging, and at treefall gaps) and along edges of forest fragments (e.g. Peñalosa 1984; Schnitzer *et al.* 2004; Zhu *et al.* 2004). With increased anthropogenic disturbances to forests worldwide, the significance and importance of lianas in forest dynamics will undoubtedly increase (Schnitzer & Bongers 2002). Therefore, while major tree species are vital to the preservation of forest habitats and their associated ecosystems, flora and fauna conservation and management should also take into account the ecology and conservation of lianas.

5.4.2. Comparison between Dietary Profile and Habitat Diversity

Among the four douc populations examined, habitat flora checklists were available for Ta Kou Nature Reserve and Cat Tien National Park. In both of these sites, Fabaceae (legume or pea family) was the dominant family based on number of taxa recorded, and I expected Fabaceae to be among the top diet families. Annonaceae (custard apple family),

Bignoniaceae (trumpet creeper family), Fagaceae (beech and oak family), and Moraceae (fig and mulberry family) were the top families recorded from the douc samples from Ta Kou, each being detected in 18 of 20 samples, while Fabaceae was among the top 10, being detected in 11 samples. In terms of the number of taxa recovered from the fecal samples, however, Fabaceae was the top family with five unique sequences, i.e. five plant taxa, detected. Similarly, for the douc samples from Cat Tien, while the top families were Anacardiaceae (cashew family) and Simaroubaceae (quassia family; bitter taste to many parts of plants), which were each detected in all seven samples, Fabaceae and Apocynaceae were both the top families in terms of the number of taxa recorded (each with three taxa) (Hypothesis 2b).

The black-shanked doucs in SBCA, Cambodia were also believed to be highly selective in their diet in relation to the species diversity within their habitat, as they were feeding on less abundant species (only 32% of identified transect tree species were utilized by the doucs; Rawson 2009), which is rather different from what was observed in the diet of Tonkin snub-nosed monkeys (**Chapter 7 on Dietary Profile of Tonkin Snub-nosed Monkeys**; whose diet was dominated by both the top family and genus within their habitat). This observation may not come as a surprise as colobine monkeys are known to be highly selective in the food species and parts of the species they consume (see Kirkpatrick 2011 and **Chapter 1.4. on Diet**).

5.4.3. Inter-population Differences

The dietary profiles between the populations were different (Hypothesis 3). As discussed in the previous section, Annonaceae, Bignoniaceae, Fagaceae and Moraceae were the most common families in the Ta Kou douc samples, while Anacardiaceae and Simaroubaceae were the most represented families in the Cat Tien douc samples. In the douc samples from Nui Chua National Park, Phyllanthaceae (leafflower family; fruits have two

seeds in each chamber) was the top family, while Annonaceae, Fabaceae, Moraceae, and Rubiaceae (coffee family) dominated the samples from Hon Heo Mountain. Only six unique plant sequences/MOTUs were found across four populations and they were Annonaceae, Lamiaceae (mint family), lamiids (clade), Lauraceae (laurel family), Moraceae and Sapotaceae (sapodilla family; trees or shrubs with milky sap) identified using the global database.

This observation may be a result of various factors, including seasonal availability in food resources and different botanical compositions between the four habitats (**Chapter 2.1. Black-shanked Douc**). Even intraspecific groups of guerezas (*Colobus guereza*) with overlapping home ranges in Kibale National Park, Uganda displayed differences in dietary profiles (i.e. intensity with which they fed on specific tree species), which could be explained by phenology and forest compositions of their core ranging areas (Harris & Chapman 2007). Proboscis monkeys (*Nasalis larvatus*) living in mangroves consume more fruits/seeds than leaves while the species living in peat swamps consumes more leaves than fruits/seeds (see Kirkpatrick 2011). The presence of sympatric langur species may also affect their dietary profiles, and particularly in Ta Kou Nature Reserve, it would be interesting to investigate the dietary profile of Annamese silvered langurs (*Trachypithecus margarita*) in order to gain a better understanding of the feeding strategies and possible resource competition between them and the black-shanked doucs (see Tran Van Bang 2013).

5.5. Summary

Diet metabarcoding of the black-shanked douc fecal samples yielded high success rates in amplification, sequencing, and replicability. It is also an efficient tool in obtaining data on food plant species consumed within a short period of time. From a total of 36 fecal samples processed, 110 unique sequences (or MOTUs) were recorded. The average number, maximum, and range of unique sequences in each fecal sample of the black-shanked douc

were the highest as compared to the Indochinese silvered langur and Tonkin snub-nosed monkey (see Fig. 7.2). Plant identifications were made by matching the diet sequences of the black-shanked doucs to the respective databases available for each of the four sites (i.e. global, local genus, and local species databases). Forty plant families were recorded, including new diet records from 18 families, 15 genera and 13 species. The top 10 families in terms of the highest number of fecal samples containing them were: Annonaceae (31 samples), Moraceae (30), Fabaceae (22), Apocynaceae (19), Bignoniaceae (18), Fagaceae (18), Menispermaceae (18), Anacardiaceae (17), Capparaceae (15), and Lauraceae (13).

Previous feeding observations revealed a dominance of Moraceae and Fabaceae based on number of taxa consumed, and both families were also among the top three families recorded in the fecal samples. Annonaceae was found in most fecal samples but was not among the top diet family recorded from the field. Additionally, all 13 plant species detected through diet metabarcoding had not been recorded as food plants from field observations, and these included a number of liana species. The black-shanked doucs in Ta Kou Nature Reserve and Cat Tien National Park were also believed to be highly selective in their diet in relation to the species diversity within their habitat, as they were feeding on less abundant species.

The dietary profiles between the four populations examined were, however, different. There was very little overlap in the food plants identified; only six sequences/MOTUs were found across four populations and they were Annonaceae, Lamiaceae, lamiids (clade), Lauraceae, Moraceae and Sapotaceae identified using the global database.

CHAPTER VI
DIETARY PROFILE OF INDOCHINESE SILVERED LANGURS

6.1. Introduction

Until recently, all silvered langurs were classified as subspecies of the silvered leaf monkey, *Trachypithecus cristatus* (see Napier 1985) which had a widespread distribution in Sundaic Southeast Asia (Malay Peninsula, Borneo, Sumatra, and associated small islands). Up to the 1990s, populations of Indochinese silvered langur (*Trachypithecus germaini*) were still almost universally considered conspecific with the silvered leaf monkey (Timmins *et al.* 2013) until it was regarded as distinct by Groves (2001, 2005). Recent morphological and molecular analyses suggest, however, that *T. germaini* should be split into two species: *T. germaini* to the west of the Mekong and Annamese silvered langur, *T. margarita* to the east (Nadler *et al.* 2005; Francis 2008; Roos *et al.* 2008, 2013; Hoang Minh Duc *et al.* 2012), although this barrier is not yet confirmed (Roos *et al.* 2013). Currently, the Indochinese silvered langur is found on mainland Southeast Asia in Cambodia (west of Mekong River), southern Lao PDR, southern Myanmar, southern Thailand, and southern tip of Vietnam.

6.2. Previous Findings

6.2.1. Feeding Observations from the Field

The feeding ecology of the Indochinese silvered langurs remains poorly understood. Some of the earliest knowledge about the diet of this species came from the ecological studies conducted by the Southern Institute of Ecology (SIE) in the Kien Luong Karst Area in southern Vietnam since 2010. With over two months of field observations in Khoe La hill, Hoang Minh Duc *et al.* (2010c) recorded more than 35 taxa of food plants and identified 26 to species (20 genera and 17 families) (Table 6.1). Most of the species recorded are singleton representatives of their families so no single family was significantly represented as food plants except for Moraceae with seven species. With 12 months of field observations on Chua

Hang hill, also in the Karst Area, Le Hong Thia *et al.* (2015) recorded 46 food plant species (34 genera and 21 families) (Table 6.1) of the Indochinese silvered langurs. The top families in terms of number of species consumed were Moraceae (12 species), Fabaceae (6), Vitaceae (5), and Euphorbiaceae (4). In Siem Reap, Cambodia, the Indochinese silvered langurs were seen descending to the ground to feed on wild mushrooms but the species was not identified (Facebook video; www.facebook.com/tailsofcambodia/videos/999657450096872/). Hence, our current understanding of the feeding ecology of the Indochinese silvered langurs is gleaned from research on the populations in Vietnam and there is little dietary information from other populations within its range in Thailand, Lao PDR, Myanmar, and Cambodia. In total, 60 food plant taxa were identified for the Indochinese silvered langurs in Vietnam (Hoang Minh Duc *et al.* 2010c; Le Hong Thia *et al.* 2015).

Table 6.1. Food plants of Indochinese silvered langurs based on field observations in Kien Luong Karst Area (Khoe La and Chua Hang hills)

	Hoang Minh Duc *et al.* 2010c (Khoe La)	**Le Hong Thia *et al.* 2015 (Chua Hang)**
Family	Species	
Acanthaceae		*Avicennia alba* *Avicennia officinalis*
Agavaceae	*Dracaena cambodiana*	*Dracaena cambodiana*
Anacardiaceae	*Lannea coromandelica*	
		Mangifera indica *Spondias pimata*
Apocynaceae		*Gymnanthera oblonga* *Sarcostemma acidum* *Tylophora* sp.
Araceae	*Scindapsus* cf. *officinalis*	*Scindapsus officinalis*
Arecaceae		*Caryota mitis*
Capparaceae	*Capparis micrantha micrantha*	
Combretaceae	*Anogeisssus acuminata* *Combretum tetralophum*	
		Terminalia triptera
Commelinaceae		*Commelina salicifolia*
Cucurbitaceae		*Luffa* sp.
Cycadaceae		*Cycas clivicola*
Dioscoreaceae	*Dioscorea bulbifera*	
Euphorbiaceae		*Bridelia monoica*
	Drypetes sp.	
	Glochidion littorale *Phyllathus reticulatus*	*Glochidion littorale* *Phyllathus reticulatus*
		Sauropus villosus
Fabaceae		*Bauhinia bracteata bracteata* *Bauhinia purpurea*

		Derris trifolia *Derris* sp.
	Derris sp. *Leucoena leucocephala*	
		Pithecellobium dulce *Tamarindus indica*
Flagellariaceae		*Flagellaria indica*
Gnetaceae		*Gnetum gnemon*
Lamiaceae		*Clerodendrum* sp. *Vitex negundo*
Loranthaceae		*Taxillus chinensis*
Malvaceae	*Grewia tomentosa*[#]	
		Sterculia stigmarota
Melastomaceae	*Memecylon caeruleum*	
Menispermaceae	*Tinospora cordifolia*	
		Tinospora crispa
Moraceae		*Ficus aurantiaca*
	Ficus benjamica	*Ficus benjamica*
		Ficus callophylla *Ficus depressa*
	Ficus hispida L.f. var. hispida	
	Ficus microcarpa *Ficus rumphii*	*Ficus microcarpa* *Ficus rumphii*
		Ficus sundaica
	Ficus superba var. japonica	*Ficus superba var. japonica*
	Ficus superba var. superba	
		Ficus tinctoria gibbosa
	Streblus asper *Streblus ilicifolius*	*Streblus asper* *Streblus ilicifolius*
Oleaceae		*Jasminum* sp.
Polypodiaceae	*Drynaria quercifolia*	*Drynaria quercifolia*
Rhamnaceae	*Ventilago cristata*	
Sapindaceae	*Allophylus glaber*	
Sonneratiaceae		*Sonneratia caseolaris*
Sterculiaceae		*Byttneria echinata*
Urticaceae	*Poikilospermum suaveolens*	
Vitaceae		*Ampelocissus hamandii* *Cayratia japonica*
	Cayratia trifolia	*Cayratia trifolia*
		Cissus hexangularis *Tetrastigma quadridens* *Tetrastigma* sp.

[#]*Grewia tomentosa* was listed under Tiliaceae in Hoang Minh Duc *et al.* (2010) but it is now under Malvaceae (The Plant List 2013a)

Both Khoe La and Chua Hang are part of the Kien Luong Karst Area, sharing similarities in habitat and vegetation components. However, the Indochinese silvered langur populations in these two hills appeared to share little similarities in their diet. Besides the observation that in both studies, Moraceae was the top family recorded with the highest number of plant taxa consumed, there was otherwise little overlap in the food plants

identified between Hoang Minh Duc *et al.* (2010c) and Le Hong Thia *et al.* (2015). Thirteen of 21 families recorded by Le Hong Thia *et al.* (2015) were not recorded in Hoang Minh Duc *et al.* (2010c). Conversely, there were seven of 17 families in Hoang Minh Duc *et al.* (2010c) that were not recorded in Le Hong Thia *et al.* (2015). Only 12 species from six families overlapped in both studies as food plants of the langurs (Table 6.1)

Several reasons may explain this dissimilarity. Khoe La hill has been under limestone quarrying pressures for cement production since 1998 (International Finance Corporation 2006) and today, more than half of the forested area in Khoe La has been denuded. The destruction of the habitat in Khoe La decreases food plant species availability, putting a constraint on the dietary diversity of the langur population in Khoe La. In contrast, Chua Hang remains relatively protected with less anthropogenic disturbance as the area is adjacent to temples and pagodas and is not being quarried. A second reason for fewer plant species recorded in Hoang Minh Duc *et al.* (2010c; 26 species) and Le Hong Thia *et al.* (2015; 46 species) may likely be the difference in survey efforts, as the earlier study was only for two months while the more recent study was conducted for one year.

The first research aim of this chapter is to describe the diet of the Indochinese silvered langurs in terms of plant taxa using DNA metabarcoding on fecal samples collected in Chua Hang, Khoe La, Lo Coc, and Mo So hills of the Kien Luong Karst Area. Results based on this approach are then compared with those documented by Hoang Minh Duc *et al.* (2010c) and Le Hong Thia *et al.* (2015) to give a preliminary assessment of the value and suitability of this DNA-based approach in relation to field observation method. Given that both studies documented Moraceae as the top family based on number of species consumed in the diet, I expect Moraceae to be among the top families in terms of the number of taxa recorded from the fecal samples collected from the Indochinese silvered langurs in Chua Hang, Khoe La, Lo Coc, and Mo So hills (Hypothesis 1).

6.2.2. Floristic Diversity in Kien Luong

The second aim of this chapter is to examine the dietary profiles of the Indochinese silvered langurs in relation to the floristic diversity within their habitats in the Kien Luong Karst Area. The Kien Luong Karst Area, together with neighboring Kien Hai forest, are characterized by four main vegetation types: limestone forests, *Melaleuca* forests and grasslands, mangroves, and coastal shrublands, during a rapid flora assessment by Nguyen Xuan Dang (2009). A total of 927 plant taxa in 563 genera and 156 families were recorded from Kien Luong-Kien Hai forests (Nguyen Xuan Dang 2009). The top 10 families in terms of the highest number of taxa recorded were: Poaceae (57 species), Cyperaceae (50), Fabaceae (50), Euphorbiaceae (40), Rubiaceae (32), Phyllanthaceae (29), Asteraceae (25), Verbenaceae (25), Moraceae (24), Asclepiadaceae (16) and Malvaceae (16).

Due to the lack of data on the abundance (based on basal area and number of stems) of the plants within each of the hills specifically and Kien Luong Karst Area generally, diet results from metabarcoding can be compared only to the floristic diversity in the Karst Area. Given that Poaceae is the top family in terms of the number of taxa recorded within the Karst Area, I expect Poaceae to be among the top families in terms of the number of taxa recorded from the fecal samples (Hypothesis 2). Finally, I aim to investigate the impact of habitat modifications to the diet of Indochinese silvered langurs. The dietary profile from a population inhabiting the less disturbed habitat (Chua Hang) will be compared to one in a more disturbed habitat (Khoe La). I expect to detect a difference in dietary profiles of the two populations of Indochinese silvered langurs (Hypothesis 3).

6.3. Diet Metabarcoding Results

6.3.1. Number of Plant Identifications

A total of 40 Indochinese silvered langur fecal samples were processed for diet metabarcoding, and all samples were retained after applying the bioinformatics filtering

criteria (see **Chapter 4.4. on Data Analysis**), indicating a low rate of amplification or sequencing error. In total, 68 unique sequences (or Molecular Operational Taxonomic Units, MOTUs) were recorded (Table 6.2). Among these, only three MOTUs were found across all four sites, and they were identified as Moraceae, Annonaceae/*Sageraea elliptica*, and Lamiaceae/*Premna*. The average number of unique sequences detected in each fecal sample was 10.6 (minimum of five and maximum of 17) and over half of the samples (62.5%) had more than 10 sequences detected (Figure 6.1). When examining the results by the sites, the average number of unique sequences detected in each fecal sample collected from Chua Hang, Khoe La, Lo Coc, and Mo So was 10.8 ± 3.0 and 9.8 ± 2.1, 13.0 ± 2.8, and 6 respectively (Table 6.2, Figure 6.2), which were not significantly different between the sites (one-way ANOVA, p=0.165, Appendix H).

Table 6.2. Number of unique sequences (MOTUs) from ISL samples across the sites

	Mo So	Lo Coc	Khoe La	Chua Hang
# of samples	1	2	11	26
Average # of MOTUs (≥90% identity score) per sample	6	13±2.8	9.8±2.1	10.8±3.0
# of MOTUs	6	21	32	47
# of MOTUs exclusive to each site	2	4	13	25
Total # of MOTUs	68			
# of shared MOTUs across all sites	3			

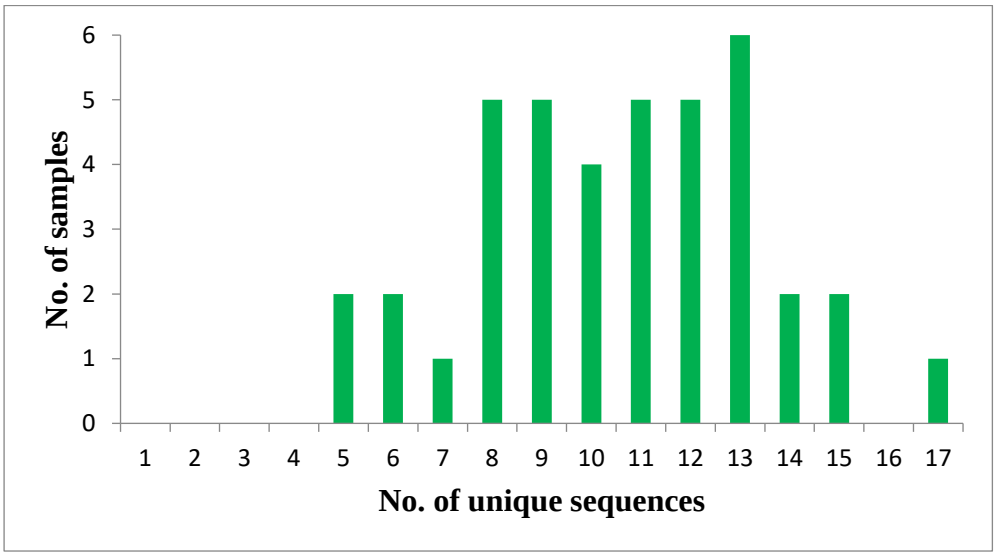


Figure 6.1. Number of unique sequences recorded in each fecal sample (ISL) from four sites (Chua Hang, Khoe La, Lo Coc, and Mo So) combined.

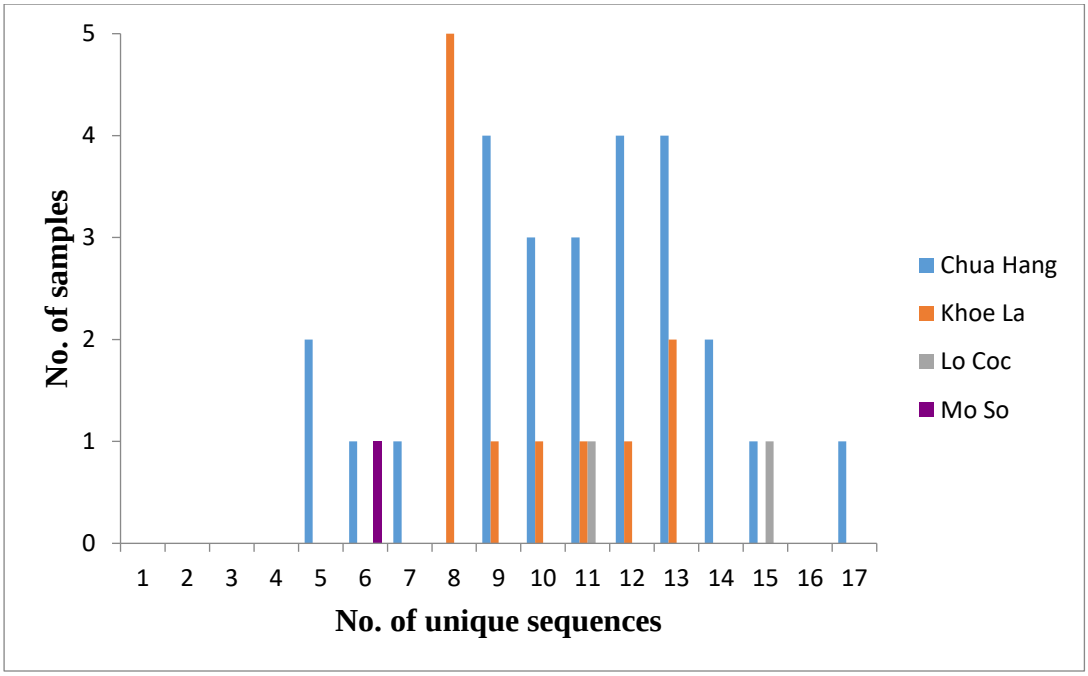


Figure 6.2. Number of unique sequences recorded in each fecal sample (ISL) from each site.

6.3.2. Taxonomic Levels of Plant Identifications

Three reference databases were built using GenBank chloroplast trnL sequences of 1) angiosperms (global database); 2) plant taxa within the genera known to occur in Kien Luong Karst Area (local genus database), and; 3) plant taxa known to occur in Kien Luong Karst Area (local species database) (following the method of Quéméré *et al.* 2013). Table 6.3 shows the taxonomic levels of plant identifications using the three different databases. Using the global reference database, 34 of 68 (50%) sequences were identified to at least family level with ≥95% best identity score, and the majority of the sequences (30.9%) were identified to family level. It also gave the highest number of incorrect identifications among the databases, with nine (13.2%) sequences assigned to genera or species not known to be found in Kien Luong or to a species with <98% identity score (following the method of de Barba *et al.* 2014). Using the local genus database, 31 of 68 (45.6%) sequences were identified to at least family level, with 20.6% of the sequences identified to genus level. Note that 90 of 563 (16.0%) genera known to occur in Kien Luong did not have sequences in GenBank (Appendix A). Finally, using the local species database, 30 of 68 (44.1%)

sequences were identified to at least family level. Majority of the sequences (35.3%) did not have an identification. Note that 569 of 927 (61.4%) taxa known to occur in Kien Luong did not have sequences in GenBank (Appendix A).

Table 6.3. Number of sequences at each level of identification using global and local databases (Kien Luong Karst Area).

Level of identification	**Global database**	**Local (Genus) database**	**Local (Species) database**	**Final identification**
NA	4	11	24 (35.3%)	22 (32.4%)
<95%	11	12	10	-
Higher than family	10	7	2	-
Family	21 (30.9%)	11	8	13
Subfamily/Tribe/Subtribe	5	4	0	5
Genus	6	14 (20.6%)	5	11
Species	2	2	17	17
Incorrect identification	9	7	2	-
Total	**68**			

NA: sequences that did not have an identification; <95%: sequences given an identification but with <95% identity score; Higher than family: sequences given an identification which is higher than family level, such as order or clade; Incorrect identification: sequences given an identification with ≥95% to a genus or ≥98% to a species that is not known to occur within Kien Luong, or to a species with 95% to <98% identity score (modified from de Barba *et al.* 2014).

6.3.3. Plant Identifications

As highlighted in Table 6.3, plant identifications of the 68 sequences varied depending on which database was used as the taxonomic sequence reference. Table 6.4a details each of the identifications using the global, local genus, and local species databases. A family/genus name was assigned if the sequence identity was ≥95% to the database and species name if the identity was ≥98% (following the method of de Barba *et al.* 2014). The finalized list of plant identifications is presented in Table 6.4b after consolidating the different taxonomic levels of identifications. For instance, sequence no. 3 was identified only to Annonaceae (family level) when using the global database and to *Sageraea* (genus level) when using the local genus database, both at 100% identity match, but the identification was refined to *Sageraea elliptica*/Annonaceae (species level) at 100% identity match when using the local species database. Therefore, the final identification for sequence no. 3 was *Sageraea*

Table 6.4a. Plant identifications for the Indochinese silvered langur samples (total samples = 40) based on global and local databases. Taxonomic assignations higher than family level are in parenthesis, while those highlighted in bold are families. Identifications are for all four sites in Kien Luong combined, unless otherwise stated.

Sequence no.	Taxon			No. of samples
	Global database	Local (Genus) database	Local (Species) database	
1	**Moraceae** (1.00)	**Moraceae (**1.00)	**Moraceae** (1.00)	38
2	NA	NA	NA	29
3	**Annonaceae** (1.00)	**Annonaceae**/*Sageraea* (1.00)	**Annonaceae**/*Sageraea elliptica* (1.00)	23
4	**Vitaceae** (1.00)	**Vitaceae** (1.00)	NA	23
5	(Lamiids) (1.00)	**Apocynaceae** (1.00)	**Apocynaceae** (1.00)	21
6	**Araceae** (1.00)	**Araceae** (1.00)	**Araceae**/*Rhaphidophora hongkongensis* (1.00)	21
7^	**Fabaceae**/Cercideae (0.98)	**Fabaceae**/*Bauhinia* (0.98)	**Fabaceae** (0.90)	15
8	**Phyllanthaceae**/*Phyllanthus talbotii* (1.00)	**Phyllanthaceae**/*Phyllanthus talbotii* (1.00)	**Phyllanthaceae**/*Phyllanthus* (0.96)	15
9	**Phyllanthaceae**/Pseudolachnostylidinae (1.00)	**Phyllanthaceae**/Pseudolachnostylidinae (1.00)	NA	14
10	**Fabaceae**/*Leucaena* (1.00)	**Fabaceae**/*Leucaena* (1.00)	**Fabaceae**/*Leucaena leucocephala* (1.00)	13
11	**Fabaceae** (0.98)	**Fabaceae**/*Derris elliptica* (0.98)	NA	13
12	**Lamiaceae**/*Premna* (1.00)	**Lamiaceae**/*Premna* (1.00)	(Pentapetalae) (0.98)	13
13	**Ebenaceae** (1.00)	**Ebenaceae**/*Diospyros* (1.00)	**Ebenaceae**/*Diospyros mollis* (0.98)	12
14	(Fabids) (1.00)	(Fabids) (1.00)	**Moraceae** (1.00)	11
15^	(Malpighiales) (0.95)	**Phyllanthaceae**/*Phyllanthus* (0.95)	NA	10
16	**Burseraceae**/*Garuda* (1.00)	**Burseraceae**/*Canarium* (0.98)	NA	9
17	**Sapindaceae** (1.00)	NA	NA	9
18	**Rhamnaceae** (0.92)	NA	NA	8
19	**Violaceae**/*Rinorea* (1.00)	NA	NA	8
20	(Cycadales) (1.00)	**Cycadaceae**/*Cycas* (1.00)	**Cycadaceae**/*Cycas* (1.00)	8
21#	(Malpighiales) (1.00)	(Malpighiales) (1.00)	**Phyllanthaceae**/*Phyllanthus* (0.96)	7
22	**Fabaceae**/Cercideae (0.94)	**Fabaceae**/*Bauhinia* (0.94)	NA	6
23#	**Malvaceae**/*Sterculia* (0.91)	**Malvaceae**/*Sterculia* (0.91)	NA	6
24#	(Lamiids) (1.00)	(Lamiids) (1.00)	**Apocynaceae**/*Gymnanthera oblonga* (1.00)	5
25	**Anacardiaceae** (1.00)	(Malvids) (0.96)	(Malvids) (0.96)	4
26^	**Menispermaceae/**Tinosporoideae (0.98)	NA	NA	4
27^	**Rubiaceae**/*Neolamarckia cadamba* (0.97)	NA	NA	4
28*	**Sapotaceae** (1.00)	**Sapotaceae** (1.00)	**Sapotaceae**/*Planchonella obovata* (1.00)	4
29#	**Annonaceae** (1.00)	**Annonaceae** (1.00)	**Annonaceae**/*Annona* (1.00)	3
30^	**Combretaceae**/*Terminalia* (1.00)	**Combretaceae**/*Terminalia* (1.00)	**Combretaceae**/*Terminalia catappa* (1.00)	3
31^	**Euphorbiaceae**/Codiaeae (1.00)	**Euphorbiaceae**/*Blachia siamensis* (1.00)	(Malpighiales) (0.94)	3
32	**Oleaceae**/*Jasminum* (1.00)	NA	NA	3
33^	**Moraceae**/*Broussonetia* (0.95)	**Moraceae**/*Broussonetia* (0.95)	**Cannabaceae** (0.95)	3
34#	**Rubiaceae** (1.00)	**Rubiaceae**/Pavetteae (1.00)	**Rubiaceae**/*Pavetta indica* (1.00)	3
35	**Rubiaceae** (1.00)	**Rubiaceae**/*Ixora ixoroides* (1.00)	**Rubiaceae** (0.98)	3
36	**Fabaceae** (0.95)	**Fabaceae**/Mimosoideae (0.95)	**Fabaceae**/*Leucaena leucocephala* (0.95)	2
37^	**Fabaceae**/*Piscidia piscipula* (1.00)	**Fabaceae**/*Derris elliptica* (0.96)	NA	2

38^	(Fabids) (0.92)	(Pentapetalae) (0.91)	**Moraceae** (0.91)	2
39^	**Loranthaceae**/*Dendrophthoe vitellina* (1.00)	**Loranthaceae**/*Dendrophthoe vitellina* (1.00)	**Loranthaceae**/*Dendrophthoe pentandra* (0.98)	2
40^	NA	NA	NA	2
41^	**Apocynaceae**/Asclepiadoideae (1.00)	**Apocynaceae**/*Dregea sinensis* (1.00)	NA	1
42[+]	**Convolvulaceae** (1.00)	**Convolvulaceae** (1.00)	**Convolvulaceae**/*Ipomoea* (1.00)	1
43[!]	**Cucurbitaceae** (1.00)	**Cucurbitaceae** (1.00)	**Cucurbitaceae**/*Coccinia grandis* (1.00)	1
44[#]	**Fabaceae** (0.98)	**Fabaceae** (0.98)	**Fabaceae** (0.98)	1
45[#]	**Fabaceae** (0.94)	**Fabaceae** (0.92)	**Fabaceae** (0.92)	1
46[#]	**Fabaceae** (0.93)	**Fabaceae**/Mimosoideae (0.93)	**Fabaceae**/*Leucaena leucocephala* (0.93)	1
47[#]	**Lamiaceae**/*Congea* (0.96)	(Lamiales) (0.92)	NA	1
48^	**Lauraceae** (1.00)	**Lauraceae** (1.00)	**Lauraceae**/*Persea americana* (1.00)	1
49^	**Malvaceae**/*Ceiba pentandra* (1.00)	**Malvaceae**/*Ceiba pentandra* (1.00)	**Malvaceae**/*Ceiba pentandra* (1.00)	1
50^	**Malvaceae**/*Sterculia* (1.00)	**Malvaceae**/*Sterculia* (1.00)	NA	1
51[+]	**Menispermaceae**/*Stephania* (1.00)	**Menispermaceae**/*Stephania* (1.00)	**Menispermaceae**/*Stephania dielsiana* (1.00)	1
52[!]	(Mesangiospermae) (0.92)	**Moraceae** (0.91)	NA	1
53^	(Fabids) (0.96)	(Fabids) (0.96)	**Moraceae** (0.96)	1
54[!]	(Pentapetalae) (0.98)	(Fabids) (0.96)	**Moraceae** (0.96)	1
55^	**Ebenaceae** (0.98)	**Ebenaceae**/*Diospyros* (0.98)	**Ebenaceae**/*Diospyros mollis* (0.98)	1
56^	(Pentapetalae) (0.94)	(Ericales) (0.94)	**Sapotaceae**/*Planchonella obovata* (0.94)	1
57[#]	(Lamiids) (0.95)	(Pentapetalae) (0.93)	**Apocynaceae** (0.93)	1
58^	(Pentapetalae) (0.92)	(Ericales) (0.92)	**Ebenaceae**/*Diospyros mollis* (0.92)	1
59^	(Fabids) (0.96)	(Fabids) (0.96)	**Moraceae** (0.96)	1
60^	(Pentapetalae) (0.94)	(Pentapetalae) (0.94)	**Moraceae** (0.94)	1
61^	(Fabids) (0.94)	(Fabids) (0.94)	**Moraceae** (0.94)	1
62[#]	**Sapindaceae** (1.00)	**Sapindaceae**/*Dimocarpus* (1.00)	**Sapindaceae**/*Dimocarpus longan* (1.00)	1
63^	**Rutaceae** (1.00)	**Rutaceae**/Aurantioideae (1.00)	**Rutaceae**/*Glycosmis pentaphylla* (1.00)	1
64[#]	**Vitaceae** (1.00)	**Vitaceae** (1.00)	NA	1
65[#]	**Dioscoreaceae**/*Dioscorea esculenta* (1.00)	**Dioscoreaceae**/*Dioscorea esculenta* (1.00)	**Dioscoreaceae**/*Dioscorea esculenta* (1.00)	1
66^	**Melastomataceae**/*Mouriri crassifolia* (0.98)	NA	NA	1
67[!]	NA	NA	NA	1
68^	NA	NA	NA	1

[*]Sequence no. 28 is the same sequence as the one identified in the positive control for *Manilkara zapota* (Sapotaceae); ^sequences only found in Chua Hang hill; [#]sequences only found in Khoe La hill; [!]sequences only found in Lo Coc hill; [+]sequences only found in Mo So hill

Table 6.4b. Finalized list of plant identifications (Kien Luong Karst Area).

Sequence no.	Taxon	No. of samples
1	**Moraceae** (1.00)	38
2	NA	29
3	**Annonaceae**/*Sageraea elliptica* (1.00)	23
4	**Vitaceae** (1.00)	23
5	**Apocynaceae** (1.00)	21
6	**Araceae**/*Rhaphidophora hongkongensis* (1.00)	21
7^	**Fabaceae**/*Bauhinia* (0.98)	15
8	**Phyllanthaceae**/*Phyllanthus* (0.96)	15
9	**Phyllanthaceae**/Pseudolachnostylidinae (1.00)	14
10	**Fabaceae**/*Leucaena leucocephala* (1.00)	13
11	**Fabaceae** (0.98)	13
12	**Lamiaceae**/*Premna* (1.00)	13
13	**Ebenaceae**/*Diospyros mollis* (0.98)	12
14	**Moraceae** (1.00)	11
15^	**Phyllanthaceae**/*Phyllanthus* (0.95)	10
16	**Burseraceae**/*Canarium* (0.98)	9
17	**Sapindaceae** (1.00)	9
18	NA	8
19	NA	8
20	**Cycadaceae**/*Cycas* (1.00)	8
21#	**Phyllanthaceae**/*Phyllanthus* (0.96)	7
22	NA	6
23#	NA	6
24#	**Apocynaceae**/*Gymnanthera oblonga* (1.00)	5
25	**Anacardiaceae** (1.00)	4
26^	**Menispermaceae/**Tinosporoideae (0.98)	4
27^	NA	4
28*	**Sapotaceae**/*Planchonella obovata* (1.00)	4
29#	**Annonaceae**/*Annona* (1.00)	3
30^	**Combretaceae**/*Terminalia catappa* (1.00)	3
31^	**Euphorbiaceae**/Codiaeae (1.00)	3
32	NA	3
33^	**Moraceae**/*Broussonetia* (0.95)	3
34#	**Rubiaceae**/*Pavetta indica* (1.00)	3
35	**Rubiaceae** (1.00)	3
36	**Fabaceae**/Mimosoideae (0.95)	2
37^	NA	2
38^	NA	2
39^	**Loranthaceae**/*Dendrophthoe pentandra* (0.98)	2
40^	NA	2
41^	**Apocynaceae**/Asclepiadoideae (1.00)	1
42+	**Convolvulaceae**/*Ipomoea* (1.00)	1
43!	**Cucurbitaceae**/*Coccinia grandis* (1.00)	1
44#	**Fabaceae** (0.98)	1
45#	NA	1
46#	NA	1
47#	NA	1
48^	**Lauraceae**/*Persea americana* (1.00)	1
49^	**Malvaceae**/*Ceiba pentandra* (1.00)	1
50^	**Malvaceae**/*Sterculia* (1.00)	1
51+	**Menispermaceae**/*Stephania dielsiana* (1.00)	1
52!	NA	1
53^	**Moraceae** (0.96)	1

54[!]	**Moraceae** (0.96)	1
55^	**Ebenaceae**/*Diospyros mollis* (0.98)	1
56^	NA	1
57[#]	NA	1
58^	NA	1
59^	**Moraceae** (0.96)	1
60^	NA	1
61^	NA	1
62[#]	**Sapindaceae**/*Dimocarpus longan* (1.00)	1
63^	**Rutaceae**/*Glycosmis pentaphylla* (1.00)	1
64[#]	**Vitaceae** (1.00)	1
65[#]	**Dioscoreaceae**/*Dioscorea esculenta* (1.00)	1
66^	NA	1
67[!]	NA	1
68^	NA	1

[*]Sequence no. 28 is the same sequence as the one identified in the positive control for *Manilkara zapota* (Sapotaceae); ^sequences only found in Chua Hang hill; [#]sequences only found in Khoe La hill; [!]sequences only found in Lo Coc hill; [+]sequences only found in Mo So hill

elliptica/Annonaceae. Similarly, for sequence no. 24, it was identified to lamiids (clade level) at 100% identity match when using the global or local genus database, but the identification was refined to *Gymnanthera oblonga*/Apocynaceae (species level) at 100% identity match when using the local species database. The final identification for sequence no. 18 was *Gymnanthera oblonga*/Apocynaceae.

The identifications were also manually checked for inaccurate identifications, such as to species or genera not known to be found in Kien Luong or Vietnam. For example, sequence no. 35 was assigned to Rubiaceae (family level) at 100% and 98% identity matches for global and local species databases respectively, but was identified as *Ixora ixoroides*/Rubiaceae (species level) at 100% match using the local genus database. However, this is likely an inaccurate identification because *Ixora ixoroides* is native to New Caledonia (see Mouly *et al.* 2009). Nonetheless, Rubiaceae was positively identified regardless of the databases and therefore, I assigned Rubiaceae as the final identification of sequence no. 35. Similarly, for sequence no. 39, it was identified as *Dendrophthoe vitellina*/Loranthaceae at 100% identity match when using either the global or local genus database, but this species is native to Australia. Using the local species database, it was identified to *Dendrophthoe*

pentandra with an identity score of 98%. Therefore, *Dendrophthoe pentandra* was assigned as the final identification of sequence no. 39 because this species is known to occur in Kien Luong and the identity was ≥98% (following the method of de Barba *et al.* 2014). In the final list of identifications (Tables 6.3 and 6.4b), 46 of 68 (67.6%) sequences were identified to at least family level, with under one-third (32.4%) of the sequences not identified.

A total of 25 families were recorded from the Indochinese silvered langur fecal samples, with Moraceae found in all 40 samples, followed by Phyllanthaceae in 34 samples, and Fabaceae in 29 samples (Figure 6.3). Twenty-five genera (*Annona*, *Bauhinia*, *Broussonetia*, *Canarium*, *Ceiba*, *Coccinia*, *Cycas*, *Dendrophthoe*, *Dimocarpus*, *Dioscorea*, *Diospyros*, *Glycosmis*, *Gymnanthera*, *Ipomoea*, *Leucaena*, *Pavetta*, *Persea*, *Phyllanthus*, *Planchonella*, *Premna*, *Rhaphidophora*, *Sageraea*, *Stephania*, *Sterculia*, and *Terminalia*) and 16 species (*Ceiba pentandra*, *Coccinia grandis*, *Dendrophthoe pentandra*, *Dimocarpus longan*, *Dioscorea esculenta*, *Diospyros mollis*, *Glycosmis pentaphylla*, *Gymnanthera oblonga*, *Leucaena leucocephala*, *Pavetta indica*, *Persea americana*, *Planchonella obovata*, *Rhaphidophora hongkongensis*, *Sageraea elliptica*, *Stephania dielsiana*, and *Terminalia catappa*) were identified. Nine families (Annonaceae, Burseraceae, Convolvulaceae, Ebenaceae, Lauraceae, Phyllanthaceae, Rubiaceae, Rutaceae and Sapotaceae), 18 genera (*Annona*, *Broussonetia*, *Canarium*, *Ceiba*, *Coccinia*, *Dendrophthoe*, *Dimocarpus*, *Diospyros*, *Glycosmis*, *Ipomoea*, *Pavetta*, *Persea*, *Phyllanthus*, *Planchonella*, *Premna*, *Rhaphidophora*, *Sageraea* and *Stephania*) and 14 species (*Ceiba pentandra*, *Coccinia grandis*, *Dendrophthoe pentandra*, *Dimocarpus longan*, *Dioscorea esculenta*, *Diospyros mollis*, *G. pentaphylla*, *Pavetta indica*, *Persea americana*, *Planchonella obovata*, *R. hongkongensis*, *Sageraea elliptica*, *Stephania dielsiana*, *T. catappa*) are new records such that there are currently 74 food plant taxa identified for Indochinese silvered langurs based on observations and metabarcoding (Appendix L).

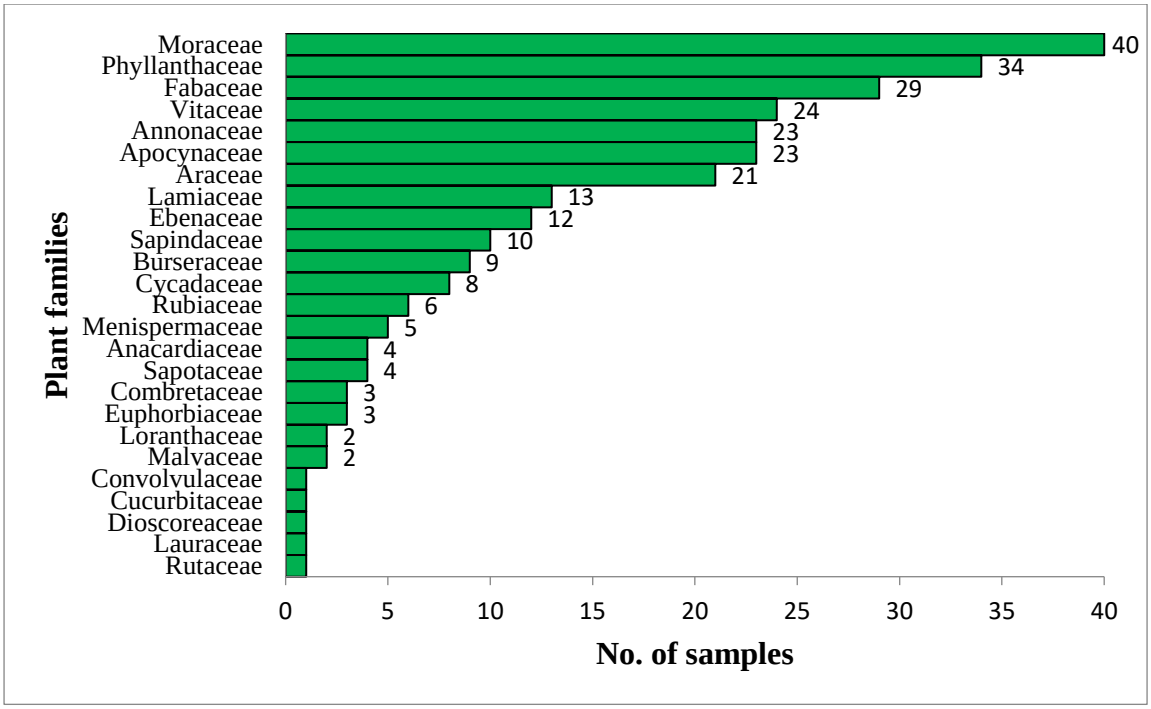


Figure 6.3. Frequency of occurrence of each plant family in the fecal samples. X-axis indicates the number of fecal samples that contained each plant families. Families with no data labels next to the bars (e.g. Rutaceae) indicate that they only occurred once in the samples. Moraceae was found in all 40 samples.

6.3.4. Effect of Habitat Disturbances

Preliminary assessment of the effect of habitat disturbances is presented here based on 11 fecal samples collected from the Indochinese silvered langur population in Khoe La hill and six fecal samples from Chua Hang hill, both sites surveyed during the same time in June 2013. Khoe La hill has been under limestone quarrying pressures for cement production since 1998 (International Finance Corporation 2006) and today, more than half of the forested area in Khoe La has been denuded, forcing the langurs to retreat into smaller limestone pockets with degraded forest cover (Hoang Minh Duc *et al.* 2010c). In contrast, Chua Hang remains relatively protected from anthropogenic disturbance as the area is adjacent to temples and pagodas. It is estimated that there are 109 Indochinese silvered langurs in Khoe La and 131 individuals in Chua Hang (Tran Van Bang *et al.* submitted).

A total of 23 plant taxa was recorded from Chua Hang samples and 32 taxa from Khoe La samples (Table 6.5). In order to examine whether there is a difference in the number

Table 6.5. Plant taxa and number of fecal samples recorded from Chua Hang and Khoe La

Taxon	Chua Hang	Khoe La
NA	6	2
Moraceae (1.00)	6	11
Vitaceae (1.00)	6	1
Araceae/*Rhaphidophora hongkongensis* (1.00)	3	
Fabaceae/*Bauhinia* (0.98)	2	
Apocynaceae (1.00)	3	7
Fabaceae (0.98)	1	
Annonaceae/*Sageraea elliptica* (1.00)	4	10
Burseraceae/*Canarium* (0.98)	2	2
Phyllanthaceae/Pseudolachnostylidinae (1.00)	1	9
Phyllanthaceae/*Phyllanthus* (0.96)	6	1
Cycadaceae/*Cycas* (1.00)	1	1
NA	5	
Lamiaceae/*Premna* (1.00)	2	7
Sapotaceae/*Planchonella obovata* (1.00)	1	1
NA	1	
Fabaceae/*Leucaena leucocephala* (1.00)	1	11
NA	4	
Moraceae/*Broussonetia* (0.95)	3	
Apocynaceae/Asclepiadoideae (1.00)	1	
Fabaceae/Mimosoideae (0.95)	1	1
Lauraceae/*Persea americana* (1.00)	1	
Moraceae (0.96)	1	
Ebenaceae/*Diospyros mollis* (0.98)		1
Anacardiaceae (1.00)		1
Rubiaceae (1.00)		1
NA		3
NA		3
NA		2
Phyllanthaceae/*Phyllanthus* (0.96)		7
NA		6
Apocynaceae/*Gymnanthera oblonga* (1.00)		5
Rubiaceae/*Pavetta indica* (1.00)		3
Annonaceae/*Annona* (1.00)		3
NA		1
NA		1
NA		1
NA		1
Sapindaceae/*Dimocarpus longan* (1.00)		1
Vitaceae (1.00)		1
Fabaceae (0.98)		1
Dioscoreaceae/*Dioscorea esculenta* (1.00)		1
Total number of taxa	23	32
Total number of plant records	62	107

of plant taxa detected between the two sites, I rarefied the Khoe La samples to match the sample size from Chua Hang using the following formula performed in Microsoft Excel version 16:

$$E(S) = \sum_{i-1}^{S} \left(1 - \left[\frac{\binom{N - N_i}{n}}{\binom{N}{n}} \right] \right)$$

where

E(S) = expected number of plant taxa in the Khoe La samples if the sample size were the same as Chua Hang's;

N = total number of plant records from the larger sample, Khoe La = 107;

N_i = number of plant records in the i^{th} taxon;

n = total number of plant records from the smaller sample, Chua Hang = 62; and

S = total number of plant taxa in Khoe La samples = 32

E(S) = 24.44, i.e. the expected number of plant taxa recorded from Khoe La would be 24.44 if the number of fecal samples collected from Khoe La had been the same as that from Chua Hang; a total of 23 plant taxa was recorded from the fecal samples collected from Chua Hang.

6.4. Discussion

Diet metabarcoding in association with next-generation sequencing has not been previously applied to fecal samples of the lutungs (*Trachypithecus* spp.). Several species of lutungs are rare and inhabit areas which are challenging for field data collection (e.g. Cat Ba langurs *T. poliocephaleus* of karst islands in Cat Ba, Vietnam) especially on their feeding ecology. In these cases, diet metabarcoding can become an important tool to complement observational research to further elucidate dietary information. Here, I demonstrated a high success rate of DNA amplification, sequencing, and replicability for the diet metabarcoding of Indochinese silvered langur fecal samples.

The level and accuracy of plant identifications to each of the unique sequences detected in the Indochinese silvered langur fecal samples varied depending on which database was used as the taxonomic reference. Among the three reference databases, the local species database might be considered as performing the best with the highest number of sequences (17 of 68, or 25%) identified to species level as compared to only two species identified in each of the global and local genus databases. Moreover, 14 of these 17 sequences were identified with high confidence; i.e. 100% identity scores to their species. Identification to species level is especially valuable to conservation such as in helping with habitat and target population recovery efforts which require the knowledge of food plant species to be planted in the habitat. The local species database also gave the lowest number of incorrect identifications (two taxa), which could in part be explained by the fact that it had the highest proportion of no identification to the sequences detected in the fecal samples (35.3% compared to 16.2% and 5.9% for local genus and global databases respectively). This in turn could be explained by the lack of barcodes of plant species found in Kien Luong (61.4%) and/or Vietnam represented in GenBank.

6.4.1. Comparison between Metabarcoding and Feeding Observations

A total of 68 unique sequences/taxa was detected in the fecal samples collected across three years from four sites, indicating an overall wide dietary selection of the Indochinese silvered langurs in Kien Luong Karst Area. Even though approximately one-third of the sequences could not be identified, 25 plant families were still recorded through metabarcoding. In comparison, 30 plant families were recorded from a total of 14 months of field research at two sites by Hoang Minh Duc *et al.* (2010c) and Le Hong Thia *et al.* (2015). This result demonstrates the efficiency and value of metabarcoding in retrieving diet information from fecal samples, which could be further increased with more DNA barcodes sequenced from the plants in the habitat in the future.

Field observations by Hoang Minh Duc *et al.* (2010c) and Le Hong Thia *et al.* (2015) documented Moraceae as the top family in terms of the number of plant species consumed by the Indochinese silvered langurs. Similarly, diet metabarcoding in my study showed that Moraceae was the dominant family in their diet as it was detected from all 40 fecal samples (Hypothesis 1), demonstrating the importance of this plant family in the diet of Indochinese silvered langurs in Kien Luong Karst Area. Within Moraceae, species of *Ficus*, or fig trees, dominated the diet as recorded by field observations (Hoang Minh Duc *et al.* 2010c; Le Hong Thia *et al.* 2015), i.e. 11 *Ficus* spp., which would lead to the expectation that the Moraceae sequences detected in the fecal samples might belong to *Ficus* species. Through metabarcoding, five unique sequences/taxa were identified to Moraceae at the family level, but could not be further refined to genus or species, i.e. no *Ficus* was identified. It could be that *Ficus* was not in the diet recovered by the fecal samples, but this is highly unlikely. Among 24 species of Moraceae found within the habitat, 11 were not represented in GenBank, and among which, nine were from *Ficus* and only two were non-*Ficus*. Therefore, at least three and up to five of the unidentified Moraceae sequences from the fecal samples belonged to *Ficus* spp. The P6 loop of trnL marker used in this study has a small size which makes it suitable for analyzing diet from degraded DNA but it has a low taxonomic resolution at the species level (Pompanon *et al.* 2012), i.e. several *Ficus* species and other Moraceae species might share the same barcode, which could further explain the lack of *Ficus* in the diet metabarcoding results.

There was little overlap in the food species identified through previous field observations and diet metabarcoding in this study; only two species, *Gymnanthera oblonga* (Apocynaceae) and *Leucoena leucocephala* (Fabaceae) overlapped as food plants for the langurs. In fact, diet metabarcoding identified 14 species, 18 genera, and nine families which were not previously recorded with field observations, hence adding information to improve

our understanding of the diet species of the Indochinese silvered langurs in the Karst Area in Vietnam.

6.4.2. Comparison between Dietary Profile and Habitat Diversity

As Poaceae was the dominant family based on the number of taxa recorded within the Karst Area, I expected that sequences corresponding to the family would be detected in the fecal samples (Hypothesis 2). However, diet metabarcoding did not identify Poaceae regardless of the databases used as the reference. This observation could be explained by the lack of reference barcodes as more than half of the taxa of Poaceae (31 of 57) recorded from the Karst Area were not represented in GenBank, or that Poaceae did not contribute to the diet of the langurs during the time of collection of fecal samples despite being the most speciose family within the habitat. Further speculations were not possible as about a third of the diet sequences could not be identified to at least family level.

6.4.3. Effect of Habitat Disturbances

Finally, I presented preliminary assessment of the impact of habitat disturbance on the dietary profiles of the Indochinese silvered langurs. The dietary profile from a population inhabiting relatively undisturbed habitat (Chua Hang) was compared to one in disturbed habitat (Khoe La), and differences in terms of number of plant taxa recovered and diversity between them were expected (Hypothesis 3). As there was a disparity in the number of fecal samples collected from both sites (Khoe La: 11 and Chua Hang: 6), I rarefied the total number of plant records from Khoe La so that they were comparable to Chua Hang. The expected number of plant taxa from Khoe La (24.44) turned out to be similar to that from Chua Hang (23) if the number of fecal samples collected from both sites had been the same. Rarefaction, however, does not account for specific taxa as it only assesses the number of taxa present in the samples, but does not take into account which taxa are represented across samples. Khoe La and Chua Hang samples might contain approximately the same number of

taxa if their sample sizes were the same, but they might have completely different plant compositions, which was unable to be assessed here.

6.5. Summary

Diet metabarcoding of the Indochinese silvered langur fecal samples yielded high success rates in amplification, sequencing, and replicability. Depending on which taxonomic reference database was used, the level and accuracy of plant identifications to each of the unique sequences detected in the fecal samples varied. Here, the local species database is considered the most optimal with the highest number of sequences identified to species level as compared to the global and local genus databases. The local species database also gave the lowest number of incorrect identifications.

A total of 68 taxa was detected in the fecal samples collected across three years from four sites, indicating an overall wide dietary selection of the Indochinese silvered langurs in Kien Luong Karst Area. Even though one-third of the sequences could not be identified, 25 plant families were still recorded through metabarcoding, demonstrating the efficiency and value of metabarcoding in retrieving diet information from fecal samples, which could be further increased as more plant species within the habitat become barcoded and available in the future.

Moraceae was the dominant family in their diet as it was detected from all 40 fecal samples. Similarly, field research at the same sites by Hoang Minh Duc *et al.* (2010c) and Le Hong Thia *et al.* (2015) documented Moraceae as the top family in terms of the number of plant species consumed by the langurs, demonstrating the importance of this plant family in their diet. However, there was otherwise little overlap in the food species identified through previous field observations and diet metabarcoding in this study as only two species, *Gymnanthera oblonga* (Apocynaceae) and *Leucoena leucocephala* (Fabaceae) overlapped as

food plants for the langurs. Metabarcoding identified 14 species, 18 genera, and nine families which were not previously recorded with field observations.

Even though Poaceae was the dominant family based on the number of taxa recorded within the Karst Area, no sequences corresponding to this family was detected in the fecal samples regardless of the databases used as the reference. This observation could be explained by the lack of reference barcodes as more than half of the taxa of Poaceae recorded from the Karst Area were not represented in GenBank, or that Poaceae, a family of grasses, did not contribute to the diet of the langurs during the time of collection of fecal samples. Lastly, impacts of habitat disturbance on dietary profiles of the langur populations could not be examined due to small sample sizes and unequal sampling efforts between the disturbed and undisturbed sites, but preliminary assessment suggested that the number of plant taxa recorded from the fecal samples in both sites was similar.

CHAPTER VII
DIETARY PROFILE OF TONKIN SNUB-NOSED MONKEYS

7.1. Introduction

The colobine genus *Rhinopithecus* (snub-nosed monkeys) includes five extant species all of which possess the characteristic upturned nostrils with the openings facing forward (Groves 2001; Geissmann *et al.* 2011). They range from southern China to the northern regions of Vietnam and Myanmar. With approximately 15,000 individuals, the golden snub-nosed monkey (*R. roxellana*) in the Chinese provinces of Gansu, Hubei, Shaanxi, and Sichuan has the largest population among the snub-nosed monkeys, followed by the black-snub-nosed monkey (*R. bieti*) with a maximum of 2,000 individuals in Tibet and Yunnan, gray snub-nosed monkey (*R. brelichi*) with 750-800 individuals in Guizhou, and Myanmar snub-nosed monkey (*R. strykeri*) with 260-330 individuals in Myanmar and 490-620 individuals in China which include some cross-boundary groups that may range between the two countries (Ma *et al.* 2014; see Yang *et al.* 2012). Lastly, the Tonkin snub-nosed monkey (*R. avunculus*) has the lowest population estimate and the most restricted distribution, with <250 individuals in two Vietnamese provinces (see Le Khac Quyet *et al.* 2015).

The feeding ecology of the Chinese snub-nosed monkeys has been examined in several areas over an extended period of time (e.g. Bleisch & Xie 1998; Bleisch *et al.* 1998), shedding light on their dietary profiles. For instance, more than 100 food plants species were identified for *R. roxellana* (Shi *et al.* 1982; Li & Shi 1986; Hu 1998), and a total of 107 food plant species from 28 families were also identified for *R. brelichi* (Xiang *et al.* 2012). Seasonal variation in their diet was detected, such as *R. bieti* consuming bamboo leaves year-round and bamboo shoots in the summer (Grueter *et al.* 2009b), and a heavy dependence on carbohydrate-rich lichens during winter by *R. bieti* (Kirkpatrick 1996; Grueter *et al.* 2009a) and *R. roxellana* (Kirkpatrick *et al.* 1999). In comparison, a comprehensive understanding of

the feeding ecology of the Myanmar snub-nosed monkey and the Tonkin snub-nosed monkey is still lacking, given that the former was only discovered by the international community in 2010 (Geissmann *et al.* 2011) and for the latter, earlier efforts and resources had to be directed toward distribution surveys and the protection of the species and its habitat (see Le Khac Quyet 2014).

7.2. Previous Findings

7.2.1. Feeding Observations from the Field

The first study on the ecology of Tonkin snub-nosed monkeys was carried out by Boonratana and Le Xuan Canh (1998) in two areas in Na Hang Nature Reserve, Tuyen Quang Province: Ta Ke and Nam Trang-Ban Bung. Having 122 contact hours (47 observational hours) over six months, 34 feeding observations were recorded but only one food plant species was identified: *Beilschmiedia* aff. *vidalli* (Lauraceae). Within my study site in Khau Ca Area in Ha Giang Province, Le Khac Quyet *et al.* (2007) identified 20 of 31 food plants to species level belonging to 22 families over 18 months from December 2004 to May 2006. During this period, Covert *et al.* (2008) carried out an ecological viability study on the same population of Tonkin snub-nosed monkeys in Khau Ca in June and July 2005 which provided additional dietary data. Hence, I discuss their results together (Table 7.1). The top families in terms of number of species consumed in both studies combined were Araliaceae (4 species), Clusiaceae (3), and Lauraceae (3). Between August 2005 and September 2006 also in Khau Ca, Dong Thanh Hai (2011) recorded 40 of 47 food plants up to species level belonging to 25 families in total. The top families in terms of number of species consumed were Annonaceae (3 species), Aquifoliaceae (3), Fagaceae (3), Myrsinaceae (3), Myrtaceae (3), and Verbenaceae (3). In total, 58 food plant taxa were identified for Tonkin snub-nosed monkeys in Tuyen Quang and Ha Giang provinces (Boonratana & Le Xuan Canh 1998; Le Khac Quyet *et al.* 2007; Covert *et al.* 2008; Dong Thanh Hai 2011).

Table 7.1. Food plants of Tonkin snub-nosed monkeys based on field observations in Khau Ca Area

	Le Khac Quyet *et al.* 2007 & Covert *et al.* 2008	**Dong Thanh Hai 2011**
Family	Species	
Aceraceae		*Acer chapaense*
	Acer tonkinensis	*Acer tonkinensis*
Anacardiaceae		*Choerospondias axillaris*
Annonaceae		*Alphonsea tonkinensis* *Limacia* sp. *Polyalthia* sp.
	Polyalthia suberosa Sp.1	
Apocynaceae	*Apocynaceae* sp. *Melodinus tourneri*	
Aquifoliaceae		*Ilex macrocarpa* *Ilex purpurea* *Ilex* sp.
Araliaceae	*Brassaiopsis* sp. *Brassaiopsis stellata*	
		Schefflera delavayi
	Schefflera palmiformis *Schefflera* aff. *velunosa*	
		Trevesia palmata
Asclepiadaceae	*Goniostemma punctatum*	
Bignoniaceae	*Rhadermachera* sp.	
Clusiaceae	*Garcinia bracteata*	
	Garcinia fagraeoides *Garcinia tinctoria*	*Garcinia fagraeoides* *Garcinia tinctoria*
Ebenaceae		*Diospyros choboensis* *Diospyros pilosula*
	Diospyros sp.	
Euphorbiaceae	*Antidesma* sp. *Sapium rotundifolium*	
Fabaceae		*Bowringia callicarpa*
	Dalbergia tonkinensis	
		Ormosia sp.
Fagaceae		*Castanopsis chinensis* *Castanopsis tonkinensis* *Quercus acustissima*
Gesneriaceae		*Anna submontana*
Icacinaceae	*Iodes seguini*	
Lauraceae		*Cryptocarya* sp.
	Lauraceae sp. *Litsea baviensis* *Litsea* sp.	
		Machilus bonii
Malvaceae	*Burretiodendron hsienmu*[#]	
Meliaceae		*Sandoricum kontape* *Toona sinensis*
Menispermaceae		*Diplospora viridiflora*
Mimosaceae	*Archidendron* sp.	
Moraceae	*Ficus* sp.	
Myrsinaceae		*Ardisia crispa* *Ardisia quinquegona* *Ardisia ramondiaeformis*
Myrtaceae		*Syzygium* sp. *Syzygium wightianum* *Syzygium zeylanicum*

Oleaceae	*Olea* sp.	
Orchidaceae	*Bulbophyllum affine*	
		Bulbophyllum pectinatum *Tropidia curculigoides*
Phyllanthaceae		*Bridelia monoica**
	*Bridelia retusa**	
Rhamnaceae		*Berchemia floribunda*
Rosaceae		*Rubus moluccana*
Rubiaceae		*Gardenia sootepesis* *Pavetta tonkinensis*
	Sp.2	
Sabiaceae	*Meliosma fordii*	
Sapindaceae	*Pometia pinnata*	
Sapotaceae		*Sarcosperma laurium*
	Sinosideroxylon wightianum	*Sinosideroxylon wightianum*
Theaceae		*Camellia sasamqua*
Ulmaceae		*Celtis sinensis*
Urticaceae	*Debregeasia squamata* f. *etuberculata*	
Verbenaceae		*Congea* sp. *Premna balansae* *Premna flavescens*
Vitaceae		*Tetrastigma gaudichaudianum*
	Tetrastigma oliviforme *Tetrastigma* sp.	

[#]*Excentrodendron tonkinensis* (Tiliaceae) was originally reported by Le Khac Quyet *et al.* (2007) and Covert *et al.* (2008) but it is now a synonym of *Burretiodendron hsienmu* (Malvaceae) (The Plant List 2013a).
**Bridelia monoica* and *B. retusa* were listed under Euphorbiaceae in Dong Thanh Hai (2011) and Le Khac Quyet *et al.* (2007) respectively but both species are currently recognized under Phyllanthaceae (Hoffmann *et al.* 2006; Govaerts 2016a).

Interestingly, even though the studies overlapped in the study period (10 months), the top five families (13 families altogether) recorded by Dong Thanh Hai (2011) were not recorded in Le Khac Quyet *et al.* (2007) and Covert *et al.* (2008). Conversely, there were 12 families in Le Khac Quyet *et al.* (2007) and Covert *et al.* (2008) that were not recorded in Dong Thanh Hai (2011). Only four species (*Acer tonkinensis*, *Garcinia fagraeoides*, *Garcinia tinctoria* and *Sinosideroxylon wightianum*) were recorded in both studies (Table 7.1).

The first research aim of this chapter is to describe the diet of the Tonkin snub-nosed monkeys in terms of plant taxa using DNA metabarcoding on fecal samples collected in Khau Ca Area. Results based on this approach are then compared with those documented by Le Khac Quyet *et al.* 2007, Covert *et al.* 2008, and Dong Thanh Hai 2011 to give a preliminary assessment of the value and suitability of this DNA-based approach in relation to field observation method. Given that independent research carried out on the same population in

almost the same time span had little overlap in dietary records, I expect, in general, a large breadth of food plants consumed by the Tonkin snub-nosed monkeys within a short period of time which could be reflected in the fecal samples that I collected from the same site over two months (Hypothesis 1).

7.2.2. Floristic Diversity and Seasonality in Khau Ca Area

The second aim of this chapter is to examine the dietary profile of the Tonkin snub-nosed monkeys in relation to the floristic diversity and abundance within their habitat in the Khau Ca Area. Khau Ca Area is located within the "Northern Indochina Subtropical Moist Forests Ecoregion", a major zoogeographic ecotone featuring high species richness for birds and mammals (Baltzer *et al.* 2001). It also belongs to the "South Chinese Floristic Province of the Indochinese Region within the Paleotropical Kingdom", a crossroad for South and East Asian floras (Takhtajan 1986). Hence, Khau Ca Area features rich fauna and flora biodiversity. The habitat is characterized by primary evergreen montane forest on limestone, secondary evergreen forest, scrub savanna, grassland, and cultivated vegetation (Nguyen Anh Duc *et al.* 2006).

A total of 532 vascular plant species belonging to 346 genera and 127 families were recorded within the 1,000 ha forest (Vu Anh Tai *et al.* 2013; numbers reported here vary from the original article after I updated the list using The Plant List 2013a). The top 10 families in terms of the highest number of taxa recorded were: Orchidaceae (40 species), Rubiaceae (26), Poaceae (18), Apocynaceae (17), Asteraceae (17), Araliaceae (14), Araceae (13), Vitaceae (13), Euphorbiaceae (12), and Moraceae (12). There were 38 families with only singleton species recorded within Khau Ca Area.

Le Khac Quyet (2014) examined six transects (1km long and 4m wide) and 30 plots (20m by 50m) from August 2009 to July 2010 which provided important data on the forest composition and phenology of Khau Ca Area. The top 10 most abundant tree families,

genera, and species based on the total number of stems are presented in Table 7.2, with Annonaceae, *Polyalthia*, and *Olea* sp. clinching the top ranks respectively. Using another indicator, the Importance Value Index which takes into account relative density and basal area, Le Khac Quyet (2014) showed that the dominant species within the habitat were *Polyalthia cerasoides* (Annonaceae), *Olea* sp. (Oleaceae), and *Burretiodendron hsienmu* (Malvaceae). In Khau Ca Area, wet season generally runs from April/May to September and dry season from October to March/April (Dong Thanh Hai 2011; Le Khac Quyet 2014). Peak production of flowers occurred from March to May, peak production of fruits from June to September, and peak production of leaves from April to August (Le Khac Quyet 2014).

Table 7.2. Top 10 most abundant tree families, genera, and species in Khau Ca based on number of stems (Le Khac Quyet 2014).

Rank	Family	Genus	Species
1	Annonaceae	*Polyalthia*	*Olea* sp.
2	Lauraceae	*Olea*	*Polyalthia cerasoides*
3	Oleaceae	*Garcinia*	*Polyalthia thorelii*
4	Euphorbiaceae	*Phoebe*	*Garcinia bracteata*
5	Clusiaceae	*Celtis*	*Burretiodendron hsienmu*^
6	Fagaceae	*Burretiodendron*[#]	*Phoebe kunstleri*
7	Sapindaceae	*Acer*	*Celtis japonica*
8	Ulmaceae	*Sapindus*	*Vernicia montana*
9	Meliaceae	*Machilus*	*Aglaia* sp.
10	Malvaceae*	*Antidesma*	*Castanopsis* sp.

*It was originally Tiliaceae because of the dominance of *Excentrodendron tonkinense*. However, *E. tonkinense* is now a synonym of *Burretiodendron hsienmu* (Malvaceae). After verifying that the rank order does not change (Appendix 2 of Le Khac Quyet 2014), I replaced Tiliaceae with Malvaceae. [#]Same as above. ^Same as above.

Given in Khau Ca Area that Annonaceae and *Polyalthia* were the dominant family and genus respectively based on abundance and that *Polyalthia cerasoides* and *Burretiodendron hsienmu* were the dominant species based on density and basal area (*Olea* sp. is excluded here as it was an unidentified species), I expect to detect sequences corresponding to these plants in the fecal samples (Hypothesis 2). Given the marked seasons and seasonality in food resources in Khau Ca Area, a difference in plant taxa consumed by the Tonkin snub-nosed monkeys would be expected. The fecal samples were collected over two months from September to October, which would correspond to the end of wet season

(September) and beginning of dry season (October) according to past climate data. However, because I do no have climate and phenological data for the time period when the fecal samples were collected, I am unable to test this hypothesis. Here, I provide a preliminary assessment of the influence of seasonality on food consumed, and expect to detect a difference in plant taxa recorded between the two months (Hypothesis 3).

7.3. Diet Metabarcoding Results

7.3.1. Number of Plant Identifications

A total of 217 Tonkin snub-nosed monkey fecal samples were processed for diet metabarcoding, and all samples were retained after applying the bioinformatics filtering criteria (see **Chapter 4.4. on Data Analysis**), indicating a low rate of amplification or sequencing error. In total, 32 unique sequences (or Molecular Operational Taxonomic Units, MOTUs) were recorded. The average number of unique sequences detected in each fecal sample was 4.1 (minimum of one and maximum of 13; 2.2 sd) and only three samples (1.4%) had more than 10 sequences detected (Figure 7.1). Most of the TSNM samples had fewer unique sequences than that detected in the black-shanked douc and Indochinese silvered langur samples (see **Chapter 5.3.1. on Number of Plant Identifications** and **Chapter 6.3.1. on Number of Plant Identifications** respectively, summarized in Figure 7.2).

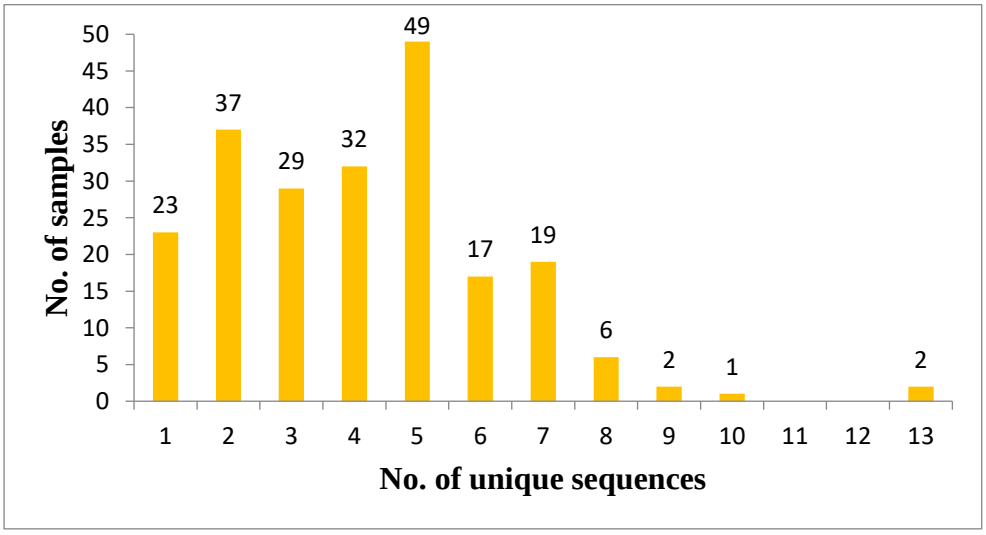


Figure 7.1. Number of unique sequences recorded in each fecal sample (TSNM).

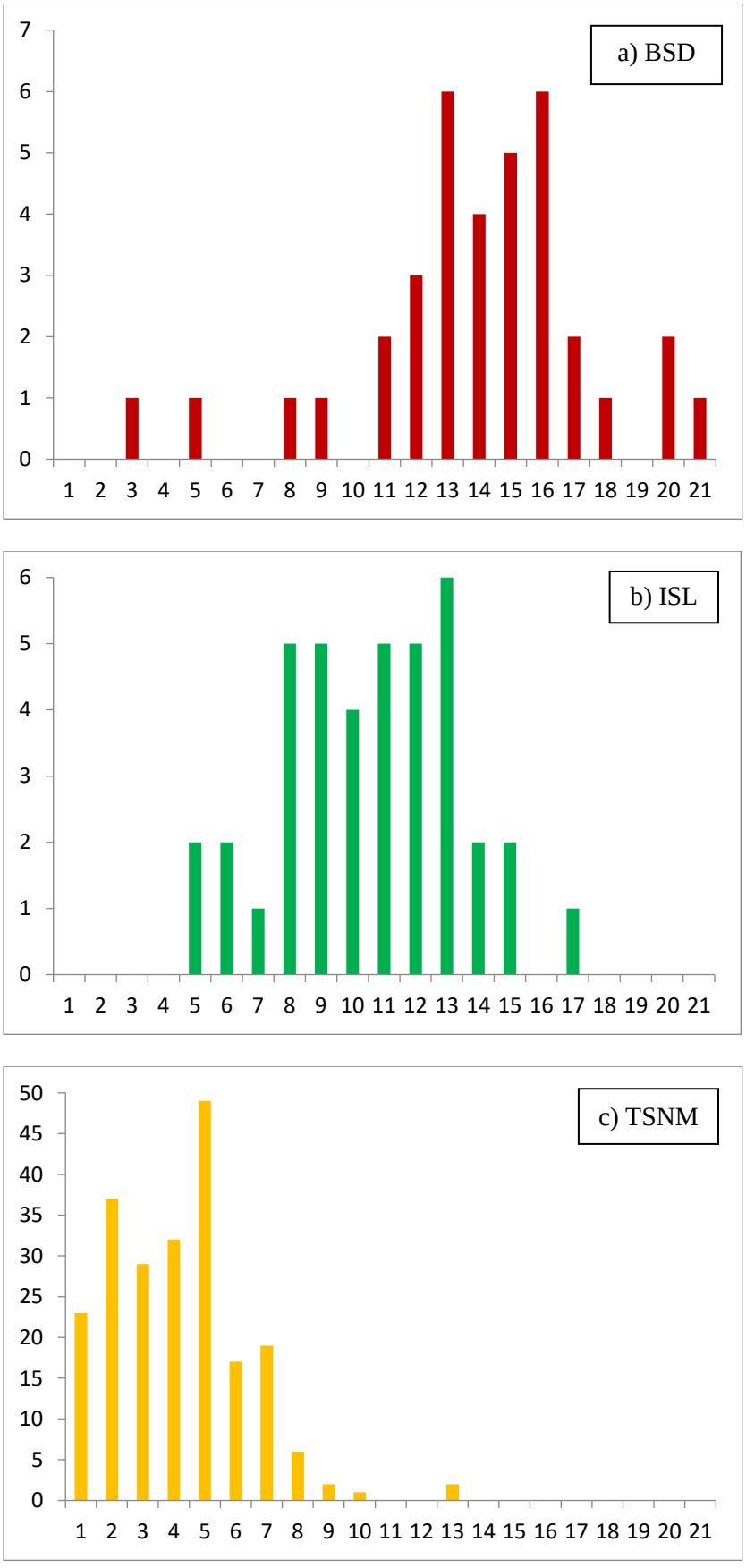


Figure 7.2. Number of unique sequences (x-axis) in each fecal sample (y-axis) for black-shanked doucs (a), Indochinese silvered langurs (b), and Tonkin snub-nosed monkeys (c).

7.3.2. Taxonomic Levels of Plant Identifications

Three reference databases were built using GenBank chloroplast trnL sequences of 1) angiosperms (global database); 2) plant taxa within the genera known to occur in Khau Ca Area (local genus database), and; 3) plant taxa known to occur in Khau Ca Area (local species database) (following the method of Quéméré *et al.* 2013). Table 7.3 shows the taxonomic levels of plant identifications using the three different databases. Using the global database, 16 of 32 (50.0%) sequences were identified to at least family level with ≥95% best identity score (Table 7.3), and the majority of the sequences (34.4%) were identified to family level. It also gave the highest number of incorrect identifications among the databases, with four (12.5%) sequences assigned to genera or species not known to be found in Khau Ca Area or to a species with <98% identity score (following the method of de Barba *et al.* 2014). Using the local genus database, 18 of 32 (56.3%) sequences were identified to at least family level, with a quarter of the sequences identified to family level. Note that 52 of 346 (15.0%) genera known to occur in Khau Ca did not have sequences in GenBank (Appendix A). Finally, using the local species database, only 11 of 32 (34.4%) sequences were identified to at least family level. The majority of the sequences (37.5%) did not have an identification. Note that 317 of 532 (59.6%) taxa known to occur in Khau Ca did not have sequences in GenBank (Appendix A).

Table 7.3. Number of sequences at each level of identification using global and local databases.

Level of identification	**Global database**	**Local (Genus) database**	**Local (Species) database**	**Final identification**
NA	1	5	12 (37.5%)	11 (34.4%)
<95%	4	7	7	-
Higher than family	7	1	0	-
Family	11 (34.4%)	8 (25.0%)	2	5
Subfamily/Tribe/Subtribe	2	2	1	2
Genus	3	7	1	8
Species	0	1	7	6
Incorrect identification	4	1	2	-
Total	**32**			

NA: sequences that did not have an identification; <95%: sequences given an identification but with <95% identity score; Higher than family: sequences given an identification which is higher than family level, such as

order or clade; Incorrect identification: sequences given an identification with ≥95% to a genus or ≥98% to a species that is not known to occur within Khau Ca Area, or to a species with 95% to <98% identity score (modified from de Barba *et al.* 2014).

7.3.3. Plant Identifications

As highlighted in Table 7.3, plant identifications of the 32 sequences varied depending on which database was used as the taxonomic sequence reference. Table 7.4a details each of the identifications using the global, local genus, and local species databases. A family/genus name was assigned if the sequence identity was ≥95% to the database and species name if the identity was ≥98% (following the method of de Barba *et al.* 2014). The finalized list of plant identifications is presented in Table 7.4b after consolidating the different taxonomic levels of identifications. For instance, sequence no. 2 was identified only to Lauraceae (family level) at 100% identity match when using the global or local genus database, but the identification was refined to *Cinnamomum burmannii*/Lauraceae (species level) at 100% identity match when using the local species database. Therefore, the final identification for sequence no. 2 was *Cinnamomum burmannii*/Lauraceae. Similarly, for sequence no. 18, it was identified only to Passifloraceae (family level) at 98% identity match when using the global or local database, but the identification was refined to *Adenia heterophylla*/Passifloraceae (species level) at 98% identity match when using the local species database. The final identification for sequence no. 18 was *Adenia heterophylla*/Passifloraceae.

The identifications were also manually checked for inaccurate identifications, such as to species or genera not known to be found in the Khau Ca Area or Vietnam. For example, while no positive identification (i.e. NA) was assigned to sequence no. 8 using the local species database, and the local genus database provided an identification to eudicotyledons (clade) at a low identity score of 90%, the sequence was identified as *Dendrobangia boliviana*/Metteniusaceae at 96% identity match using the global database. However, this is

Table 7.4a. Plant identifications for the Tonkin snub-nosed monkey samples (total samples=217) based on global and local databases. Taxonomic assignations higher than family level are in parenthesis, while those highlighted in bold are families.

Sequence no.	Taxon			No. of samples
	Global database	Local (Genus) database	Local (Species) database	
1	**Annonaceae** (1.00)	**Annonaceae**/*Polyalthia* (1.00)	**Annonaceae** (0.91)	217
2	**Lauraceae** (1.00)	**Lauraceae** (1.00)	**Lauraceae**/*Cinnamomum burmannii* (1.00)	151
3*	**Sapotaceae** (1.00)	**Sapotaceae**/*Mimusops capuronii* (1.00)	**Theaceae**/*Camellia caudata* (0.92)	130
4	(Magnoliidae) (0.98)	**Lauraceae** (0.96)	**Lauraceae**/*Cinnamomum burmannii* (0.96)	110
5	**Annonaceae** (0.96)	**Annonaceae**/*Polyalthia* (0.96)	NA	87
6	(Mesangiospermae) (0.91)	NA	NA	49
7	**Vitaceae** (1.00)	**Vitaceae** (1.00)	**Vitaceae** (1.00)	41
8	**Metteniusaceae**/*Dendrobangia boliviana* (0.96)	(Eudicotyledons) (0.90)	NA	23
9	(Pentapetalae) (1.00)	**Ebenaceae**/*Diospyros* (0.98)	**Theaceae**/*Camellia caudata* (0.96)	21
10	**Annonaceae**/*Isolona* (1.00)	(Magnoliales) (0.96)	**Annonaceae** (0.94)	9
11	**Orchidaceae**/Epidendroideae (1.00)	**Orchidaceae**/Epidendroideae (1.00)	**Orchidaceae**/*Bulbophyllum affine* (1.00)	6
12	**Araliaceae** (1.00)	**Araliaceae** (1.00)	**Araliaceae** (1.00)	5
13	(Magnoliidae) (0.96)	**Annonaceae**/*Polyalthia* (0.94)	NA	4
14	**Rubiaceae** (1.00)	**Rubiaceae**/Gardenieae (1.00)	**Rubiaceae**/Cinchonoideae (0.95)	4
15	(Laurales) (0.98)	**Lauraceae** (0.98)	**Lauraceae**/*Cinnamomum burmannii* (0.98)	4
16	(Magnoliophyta) (0.96)	(Magnoliidae) (0.94)	**Lauraceae**/*Cinnamomum burmannii* (0.94)	4
17	**Fabaceae**/*Senegalia tenuifolia* (1.00)	**Fabaceae**/*Acacia* (0.98)	NA	3
18	**Passifloraceae** (0.98)	**Passifloraceae** (0.98)	**Passifloraceae**/*Adenia heterophylla* (0.98)	3
19	**Sapindaceae** (1.00)	**Sapindaceae**/*Pometia pinnata* (1.00)	**Sapindaceae**/*Pometia pinnata* (1.00)	3
20	(Pentapetalae) (0.94)	(Ericales) (0.94)	NA	3
21	(Laurales) (0.98)	**Magnoliaceae**/*Magnolia* (0.96)	**Lauraceae**/*Cinnamomum burmannii* (0.94)	2
22	**Bignoniaceae** (1.00)	**Lamiaceae** (0.96)	**Bignoniaceae**/*Oroxylum indicum* (0.93)	2
23	**Cucurbitaceae**/*Gynostemma pentaphyllum* (1.00)	NA	NA	2
24	**Rhamnaceae** (0.92)	NA	NA	2
25	(Pentapetalae) (0.96)	(Pentapetalae) (0.94)	(Campanulids) (0.92)	2
26	**Apocynaceae**/*Tabernaemontana* (1.00)	**Apocynaceae**/*Tabernaemontana* (1.00)	**Apocynaceae**/*Alstonia scholaris* (0.98)	1
27	**Araceae** (1.00)	**Araceae** (1.00)	**Araceae**/*Rhaphidophora hookeri* (1.00)	1
28	**Oleaceae**/*Jasminum* (1.00)	**Oleaceae**/*Jasminum* (1.00)	**Oleaceae**/*Jasminum* (1.00)	1
29	**Phyllanthaceae**/Pseudolachnostylidinae (1.00)	NA	NA	1
30	**Rosaceae**/*Prunus* (1.00)	**Rosaceae**/Maleae (0.94)	NA	1
31	(Mesangiospermae) (0.93)	(Gentianales) (0.91)	NA	1
32	NA	NA	NA	1

*Sequence no. 3 is the same sequence as the one identified in the positive control for *Manilkara zapota* (Sapotaceae).

Table 7.4b. Finalized list of plant identifications (Khau Ca Area).

Sequence no.	Taxon	No. of samples
1	**Annonaceae**/*Polyalthia* (1.00)	217
2	**Lauraceae**/*Cinnamomum burmannii* (1.00)	151
3*	**Sapotaceae** (1.00)	130
4	**Lauraceae** (0.96)	110
5	**Annonaceae**/*Polyalthia* (0.96)	87
6	NA	49
7	**Vitaceae** (1.00)	41
8	NA	23
9	**Ebenaceae**/*Diospyros* (0.98)	21
10	NA	9
11	**Orchidaceae**/*Bulbophyllum affine* (1.00)	6
12	**Araliaceae** (1.00)	5
13	NA	4
14	**Rubiaceae**/Gardenieae (1.00)	4
15	**Lauraceae**/*Cinnamomum burmannii* (0.98)	4
16	NA	4
17	**Fabaceae**/*Acacia* (0.98)	3
18	**Passifloraceae**/*Adenia heterophylla* (0.98)	3
19	**Sapindaceae**/*Pometia pinnata* (1.00)	3
20	NA	3
21	**Magnoliaceae**/*Magnolia* (0.96)	2
22	**Bignoniaceae** (1.00)	2
23	NA	2
24	NA	2
25	NA	2
26	**Apocynaceae**/*Tabernaemontana* (1.00)	1
27	**Araceae**/*Rhaphidophora hookeri* (1.00)	1
28	**Oleaceae**/*Jasminum* (1.00)	1
29	**Phyllanthaceae**/Pseudolachnostylidinae (1.00)	1
30	**Rosaceae**/*Prunus* (1.00)	1
31	NA	1
32	NA	1

*Sequence no. 3 is the same sequence as the one identified in the positive control for *Manilkara zapota* (Sapotaceae).

likely an inaccurate identification because *Dendrobangia* is native to Central and South America (Govaerts 2016b) and not known to occur in Vietnam, and that the taxon and genus of sequence no. 8 did not have representative barcodes in GenBank. Therefore, no identification was assigned for sequence no. 8. Similarly, for sequence no. 10, it was identified as *Isolona*/Annonaceae at 100% identity match, but this genus is only found in Africa and Madagascar (see Couvreur *et al.* 2006). Using the local species database, it was identified to Annonaceae with a low identity score of 94% and the local genus database provided an identification to Magnoliales (order level) at 96%. The identification to order level does not value-add to this research nor conservation efforts in a broader application

sense, and so no identification was assigned for sequence no. 10. In the final list of identifications (Tables 7.3 and 7.4b), 21 of 32 (65.6%) sequences were identified to at least family level, with one-third of the sequences not identified.

A total of 18 families were recorded from the Tonkin snub-nosed monkey fecal samples, with Annonaceae found in all 217 samples, followed by Lauraceae in 152 samples, and Sapotaceae in 130 samples (Figure 7.3). Twelve genera (*Acacia*, *Adenia*, *Bulbophyllum*, *Cinnamomum*, *Diospyros*, *Jasminum*, *Magnolia*, *Polyalthia*, *Pometia*, *Prunus*, *Rhaphidophora*, and *Tabernaemontana*) and five species (*Adenia heterophylla*, *B. affine*, *C. burmannii*, *Pometia pinnata* and *R. hookeri*) were identified. Three families (Araceae, Magnoliaceae and Passifloraceae), eight genera (*Acacia*, *Adenia*, *Cinnamomum*, *Jasminum*, *Magnolia*, *Prunus*, *Rhaphidophora*, and *Tabernaemontana*) and three species (*A. heterophylla*, *C. burmannii*, and *R. hookeri*) are new records such that there are currently 61 food plant taxa identified for TSNM based on observations and metabarcoding (Appendix N).

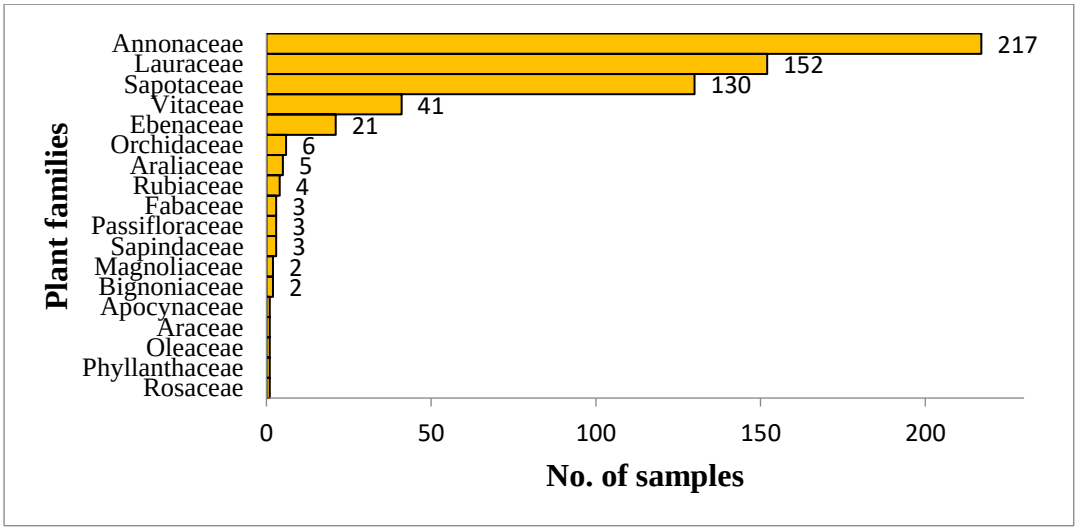


Figure 7.3. Frequency of occurrence of each plant family in the fecal samples. X-axis indicates the number of fecal samples that contained each of the plant families. Families with no data labels next to the bars (e.g. Rosaceae) indicate that they only occurred once in the samples. Annonaceae was found in all 217 samples.

When compared with the samples of black-shanked douc and Indochinese silvered langur, the TSNM samples had the lowest number of plant families detected (see **Chapter**

5.3.3.5. on Summary and **Chapter 6.3.3. on Plant Identifications** respectively, summarized below in Figure 7.4).

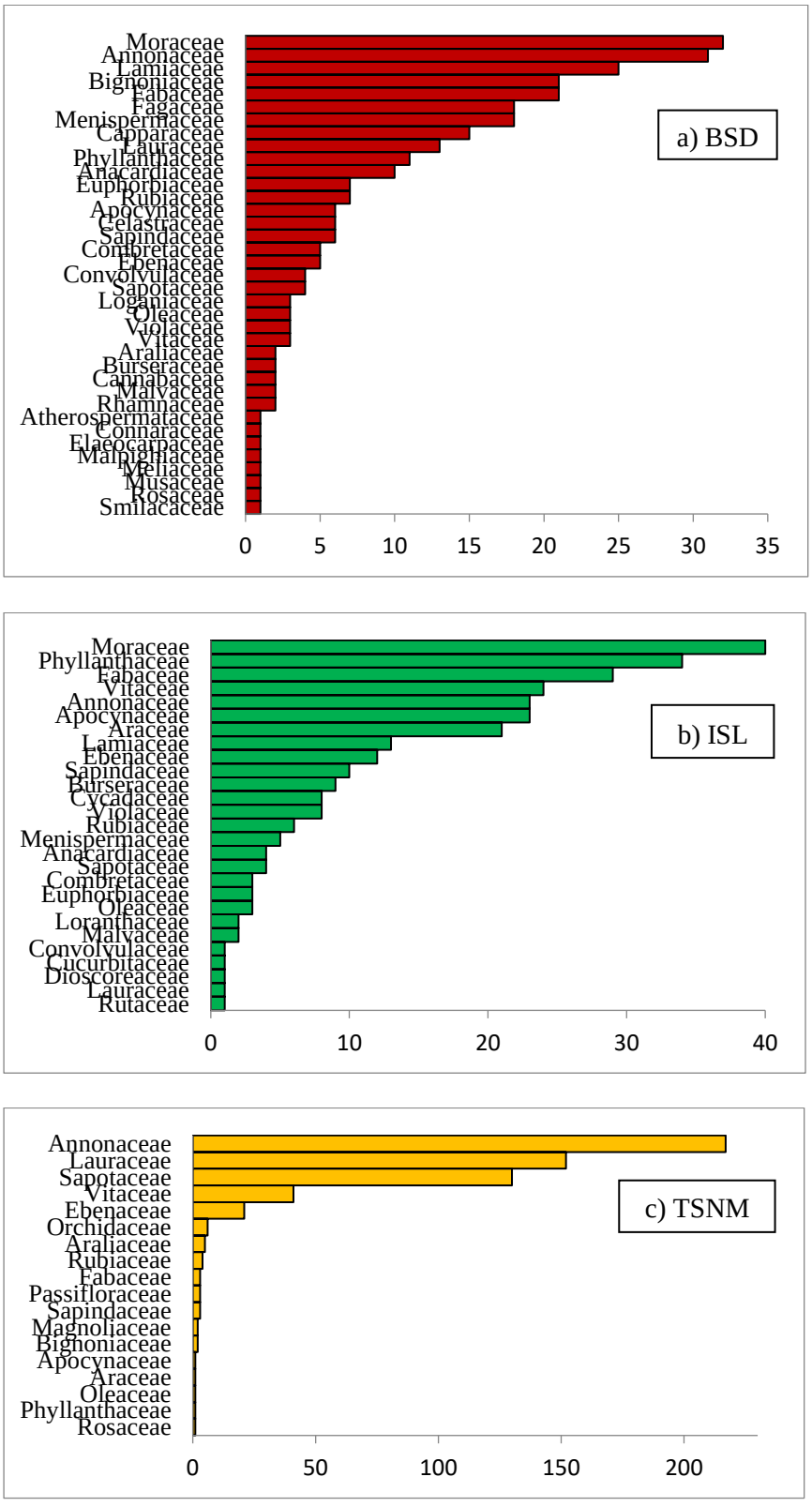


Figure 7.4. Frequency of occurrence (x-axis) of each plant family (y-axis) in the fecal samples of black-shanked doucs (a), Indochinese silvered langurs (b), and Tonkin snub-nosed monkeys (c).

7.3.4. Diet Transition between Seasons

Table 7.5 highlights the plant taxa that were recorded in the fecal samples collected from 6 September to 31 October 2014, separated into four two-week periods. Eight taxa were consistently detected in the samples throughout the two-month period: two from *Polyalthia* (**Annonaceae**), two from **Lauraceae**, one each from **Sapotaceae** and **Vitaceae**, and two unidentified taxa. In order to examine whether there is significant relationship between the period of collection (the end of wet season to the start of dry season) and the plant taxa recorded in the fecal samples, a Chi-Square Test of Independence was performed using the total sequence reads of each plant taxon in the samples collected in each of the two-week periods (Table 7.5), where:

"*Null hypothesis: There is no significant relationship between the period of collection and the plant taxa recorded in the fecal samples.*"

"*Alternative hypothesis: There is a significant relationship between the period of collection and the plant taxa recorded in the fecal samples.*"

Sequence reads were log transformed to attenuate the effect of extreme values. The data were analyzed using Microsoft Excel version 16, with the degrees of freedom (df) = (number of rows − 1) x (number of columns − 1) = 93. The Chi-Square test statistic is 148.494 while the critical value with df between 90-100 is between 128.299 and 140.169 at 99.5% significance level. As 148.494 > 140.169, the null hypothesis is rejected and the plant taxa detected in fecal samples were dependent on which time period they were collected at 99.5% significance level. Linking these differences to the changing seasons is challenging, however, since I do not have climatic data for the actual months in which fecal samples were collected.

Table 7.5. Plant taxa and their sequence reads (libraries 1 and 2 combined) in the fecal samples collected during each two-week period.

Taxon	1st (6-19Sep)	2nd (20Sep-3Oct)	3rd (4-17Oct)	4th (18-31Oct)
Annonaceae/*Polyalthia* (1.00)	1,368,895	1,714,644	1,297,637	1,118,800
Lauraceae/*Cinnamomum burmannii* (1.00)	109,945	46,343	24,583	7,934
Sapotaceae (1.00)	197,796	4,071	52,147	35,864
Lauraceae (0.96)	19,912	9,340	7,311	2,215
Annonaceae/*Polyalthia* (0.96)	13,608	7,557	5,151	1,208
NA	4,645	3,803	2,076	8,499
Vitaceae (1.00)	4,028	212	2,724	4,147
NA	13,414	2,488	0	0
Ebenaceae/*Diospyros* (0.98)	10,092	0	0	0
NA	604	0	919	224
Orchidaceae/*Bulbophyllum affine* (1.00)	1,715	452	0	0
Araliaceae (1.00)	613	0	0	0
NA	75	193	142	191
Rubiaceae/Gardenieae (1.00)	0	0	0	1,135
Lauraceae/*Cinnamomum burmannii* (0.98)	718	485	0	0
NA	565	0	0	0
Fabaceae/*Acacia* (0.98)	691	0	0	0
Passifloraceae/*Adenia heterophylla* (0.98)	409	70	0	0
Sapindaceae/*Pometia pinnata* (1.00)	1,140	0	0	0
NA	509	0	0	0
Magnoliaceae/*Magnolia* (0.96)	361	0	0	0
Bignoniaceae (1.00)	176	0	0	188
NA	0	0	0	1,240
NA	895	211	0	0
NA	460	0	0	0
Apocynaceae/*Tabernaemontana* (1.00)	0	407	0	0
Araceae/*Rhaphidophora hookeri* (1.00)	125	0	0	0
Oleaceae/*Jasminum* (1.00)	0	1,968	0	0
Phyllanthaceae/Pseudolachnostylidinae (1.00)	0	0	0	114
Rosaceae/*Prunus* (1.00)	0	1,156	0	0
NA	0	367	0	0
NA	0	289	0	0

7.4. Discussion

Diet metabarcoding in association with next-generation sequencing has not been previously applied to fecal samples of the snub-nosed monkeys (*Rhinopithecus* spp.). Its application to elucidate dietary information may not be as valuable for the relatively better-studied Chinese snub-nosed monkeys, but it can be useful for the less-studied and rarer Myanmar snub-nosed monkey and Tonkin snub-nosed monkey (TSNM). Here, I demonstrated a high success rate of DNA amplification, sequencing, and replicability for the diet metabarcoding of TSNM fecal samples.

The level and accuracy of plant identifications to each of the unique sequences detected in the TSNM fecal samples varied depending on which database was used as the

taxonomic reference. Among the three reference databases, the local genus database performed the best with the highest proportion of sequences (56%) identified to at least family level and with the lowest number of incorrect identifications (one taxon). Local species database performed the worst, which is likely due to the lack of barcodes of species found in Khau Ca (~60%) and/or Vietnam represented in GenBank. In this case, many of the diet sequences would be returned with no identification if a local species database was used as a reference. The global database was also not ideal as some of the identifications were to genera or species not known to be found in Vietnam.

7.4.1. Comparison between Metabarcoding and Feeding Observations

The number of sequences detected in each fecal sample was low which was unexpected (Hypothesis 1) given that the TSNMs in Khau Ca Area appeared to display a wide dietary selection based on field observations and more than 200 fecal samples were collected for diet metabarcoding. Consequently, only 32 plant taxa were recorded. Given that 11 of 32 taxa could not be identified, a total of 18 families were identified from the remaining sequences.

A majority of the families detected through metabarcoding were also recorded as food plants through field observations, demonstrating a general concordance between these two methods. Specifically, *Bulbophyllum affine* and *Pometia pinnata* (Sapindaceae) were recorded using both methods. *Bulbophyllum affine* is a species of orchid in the family Orchidaceae and *Pometia pinnata* is a fairly large hardwood timber tree (>25m in height) in the family Sapindaceae which is also considered as a medicinal plant (Vu Anh Tai *et al.* 2013).

Additionally, diet metabarcoding identified three species (*Adenia heterophylla*, *Cinnamomum burmannii* and *Rhaphidophora hookeri*), five genera (*Acacia*, *Jasminum*, *Magnolia*, *Prunus* and *Tabernaemontana*) and three families (Araceae, Magnoliaceae and

Passifloraceae) which were not previously recorded with field observations. Both *A. heterophylla* (Passifloraceae) and *R. hookeri* (Araceae) are perennial climbers which are considered to have medicinal properties (Vu Anh Tai *et al.* 2013). *Cinnamomum burmannii* (Lauraceae) is an evergreen tree whose bark is used as the spice cinnamon.

7.4.2. Comparison between Dietary Profile and Habitat Diversity

As Annonaceae and *Polyalthia* were the dominant family and genus respectively based on abundance in Khau Ca Area (Le Khac Quyet 2014), I hypothesized that sequences corresponding to the family and genus would be detected in the fecal samples (Hypothesis 2). Diet metabarcoding found that not only were both Annonaceae and *Polyalthia* sequences retrieved from the fecal samples, but that they were also the dominant family and genus in the TSNM diet at the population level as all the individuals sampled (i.e. all 217 fecal samples) fed on *Polyalthia*/Annonaceae plants. The second most dominant family and genus in Khau Ca was Lauraceae and *Olea* respectively. Lauraceae was also the second most dominant family in the TSNM diet in terms of the number of fecal samples with a Lauraceae sequence. However, it was *Cinnamomum*, instead of *Olea*, that was the second most dominant genus among the samples.

Nonetheless, the results from diet metabarcoding demonstrated a positive selection of the most abundant plants (in terms of number of stems) within the habitat, which is similar to that reported in the field observations of another colobine monkey, the white-thighed surili (*Presbytis siamensis*) at Kuala Lompat in Malaysia. Almost half of its diet (~46%) came from the top five most common tree families (totaling 49% in terms of basal area) within the habitat (Davies *et al.* 1988). In contrast, only 7% of the diet of the maroon langur (*P. rubicunda*) at Sepilok in Borneo came from the top five most common tree families (83% of basal area) (Davies *et al.* 1988). Even though colobine monkeys are generally known to be picky eaters (Kirkpatrick 2011), their diet can vary between populations, species, and sites,

and so a long-term study between populations of a species is required in order to provide a comprehensive picture of its feeding ecology.

Interestingly, even though *Cinnamomum* was the second most common genus in the fecal samples, it was among the least abundant within Khau Ca, and *C. burmannii*, the most dominant species recorded in the fecal samples, was not recorded within the plots and transects (Le Khac Quyet 2014). Based on density and basal area, Le Khac Quyet (2014) found *Polyalthia cerasoides* and *Burretiodendron hsienmu* to be the dominant species in Khau Ca Area. Both of these species were not identified through metabarcoding, likely because either they were not eaten by the TSNMs sampled, or their sequences cannot be identified due to the lack of barcodes of these two species in GenBank database.

7.4.3. Diet Transition between Seasons

Seasonal transition in diet can be interpreted as a shift in food choices from one plant part to another such as predominantly mature leaves during the dry season to young leaves or fruits when they become available, as observed in capped langurs (*Trachypithecus pileatus*; Stanford 1991) in Madhupur National Park, Bangladesh, or a switch between plant species in different seasons, especially if the animals select the same plant parts. The golden snub-nosed monkeys (*Rhinopithecus roxellana*) in Shennongjia Nature Reserve, China consumed buds or young leaves from *Cornus controversa* (family Cornaceae) during the spring and fall, but did not select this species in the summer, and consumed buds or young leaves from *Albizia julibrissin* (Leguminosae), *Tilia oliveri* (Malvaceae) and *Zanthoxylum bungeanum* (Rutaceae) in the summer and did not select these species any other time (Li 2001).

Given that a number of previous work documented seasonality in climate and food availability in Khau Ca Area (e.g. Dong Thanh Hai 2011 and Le Khac Quyet 2014), I expected to detect a difference in plant taxa consumed by the Tonkin snub-nosed monkeys. I found a significant difference in the dietary profiles of the monkeys in terms of plant taxa

detected in the fecal samples collected in September and October. Even though the plant taxa detected were highly dependent on which time period the samples were collected, an influence of seasonality, however, could not be tested because I did not collect climate and phenological data during the same time. Note that diet metabarcoding was unable to provide information on the plant parts consumed.

Based on data collected in 2010, peak production of flowers occurred from March to May, peak production of fruits from June to September, and peak production of leaves from April to August (Le Khac Quyet 2014). I collected the TSNM fecal samples over two months from September to October 2014, which might not have coincided with the leafing and flowering periods but occurred toward the end of peak fruiting period. Nine taxa including *Pometia pinnata*/Sapindaceae, *Rhaphidophora hookeri*/Araceae, *Acacia*/Fabaceae, *Diospyros*/Ebenaceae, *Magnolia*/Magnoliaceae, and Araliaceae were only detected in September (probably the end of wet season), while three taxa including Phyllanthaceae and Rubiaceae were only detected in October (probably the beginning of dry season). The TSNMs are known to feed on ripe and unripe fruits of *Pometia pinnata* (Le Khac Quyet *et al.* 2007) and it was likely that they were feeding on the fruits of this species before the end of the fruiting period.

On the other hand, *Polyalthia* of the custard apple family Annonaceae was positively selected throughout the two months. At least three species are currently found within Khau Ca Area: *P. cerasoides*, *P. nemoralis*, and *P. suberosa*, but a positive identification to either one of these species was not possible. Based on field observations, the TSNMs fed on the flowers of *P. suberosa* (Covert *et al.* 2008) and young leaves and flowers of an unidentified *Polyalthia* sp. (Dong Thanh Hai 2011). *Polyalthia suberosa* is also known to flower almost year-round and to bear fruits from June to December (Li 2011). It would be interesting to follow up on the species identity of this *Polyalthia* sequence, to examine the phenology of *P.*

suberosa and other species of the genus within Khau Ca, and to determine which plant parts are selected by the monkeys. In addition, having fecal samples representing a wider time frame would also improve this analysis.

7.5. Summary

Diet metabarcoding of the Tonkin snub-nosed monkey fecal samples yielded high success rates in amplification, sequencing, and replicability. It is also an efficient tool in obtaining data on food plant species consumed within a short period of time. While Le Khac Quyet *et al.* (2007) identified 20 of 31 food plants to species over 18 months of field work and Dong Thanh Hai (2011) recorded 40 of 47 food plants to species over 14 months of field work, diet metabarcoding of 217 fecal samples collected over two months in this current study detected 32 plant taxa. One disadvantage of metabarcoding at this time is the lack of taxonomic resolution at the species level due to insufficient representative barcodes of plant taxa found within Khau Ca Area. Its value will undoubtedly increase as more plant species within the habitat become barcoded and available in the database. As such, an important step toward a large-scale and comprehensive examination of the diet of Tonkin snub-nosed monkeys at the individual and population levels over an extended period of time should begin with the barcoding of plant taxa within Khau Ca Area, followed by exploring the use of both field observations and diet metabarcoding.

A total of 18 plant families was identified through diet metabarcoding, with the dominant taxon found in all fecal samples belonging to *Polyalthia* of the family Annonaceae. *Polyalthia* and Annonaceae were also the dominant genus and family respectively within Khau Ca Area (Le Khac Quyet 2014). Metabarcoding also identified new diet records which were previously not recorded with field observations, which included three families (Araceae, Magnoliaceae and Passifloraceae), five genera (*Acacia*, *Jasminum*, *Magnolia*, *Prunus* and *Tabernaemontana*) and three species (*Adenia heterophylla*, *Cinnamomum burmannii* and

Rhaphidophora hookeri). It was also found that the food plant species selected by TSNMs were dependent on which time period it was, but the identification of the plant parts consumed was not possible via metabarcoding.

CHAPTER VIII
CONCLUSION AND CONSERVATION IMPLICATIONS

This dissertation examined the genetic variability and diet metabarcoding of three highly threatened species of colobine primates using fecal samples collected across six sites in Vietnam: the black-shanked douc (*Pygathrix nigripes*) from Ta Kou Nature Reserve, Cat Tien National Park, Nui Chua National Park and Hon Heo Mountain, the Indochinese silvered langur (*Trachypithecus germaini*) from the Kien Luong Karst Area, and the Tonkin snub-nosed monkey (*Rhinopithecus avunculus*) from the Khau Ca Area. In this final chapter, I summarized the major findings and their conservation implications, and suggested recommendations for future studies.

8.1. Genetic Variability

In Chapter 3, I extracted, amplified, and sequenced the mitochondrial DNA hypervariable region I (HV-I) and entire d-*loop* from fecal samples of the three colobines using designed primers. A subset of the Tonkin snub-nosed monkey samples was also prepared and sent for direct shotgun sequencing in order to obtain full mitochondrial genomes. Overall, the genetic variability in the black-shanked doucs was high, followed by the Indochinese silvered langurs, with the lowest variability found in the Tonkin snub-nosed monkeys.

Even though the mitochondrial variability in the black-shanked doucs from Ta Kou Nature Reserve, Cat Tien National Park, Nui Chua National Park and Hon Heo Mountain were high whether examined together or separately, there was likely a lack of recent gene flow between all the populations as there were no shared genetic haplotypes between the four sites. In the Indochinese silvered langurs, genetic variability was also relatively high. However, each of the four isolated subpopulations in Kien Luong Karst Area exhibited low genetic variability when considered separately. Only Khoe La hill, the site under the most

intense anthropogenic disturbance through mining activities of the four sites, contained haplotypes that were also found in the other sites. Extirpation of the Khoe La population would mean that the remaining haplotypes would become isolated from each other. Lastly, all HV-I sequences and mitochondrial genomes of the Tonkin snub-nosed monkeys in Khau Ca Area showed zero variability, which is the lowest mitochondrial genetic variability ever reported for any primate species in the wild (Ang *et al.* 2016).

8.2. Diet Metabarcoding

In Chapters 5-7, I characterized the dietary profiles of the three colobine species using a DNA-based approach combining barcoding and next-generation sequencing technologies. Diet metabarcoding of the fecal samples yielded high success rates in amplification, sequencing, and replicability. From a total of 36 black-shanked douc fecal samples processed, 110 unique diet sequences (or MOTUs) were recorded, which was the highest as compared to the Indochinese silvered langur and Tonkin snub-nosed monkey. Forty Indochinese silvered langur samples were processed for diet metabarcoding and 68 unique sequences were retained. For Tonkin snub-nosed monkey, 32 unique sequences were recovered from 217 samples. The average number, maximum, and range of unique sequences in each fecal sample of the black-shanked douc were also the highest as compared to the other two colobines.

A total of 40 plant families was recorded in the black-shanked douc samples, with Annonaceae being the most common family in terms of the highest number of fecal samples containing them, followed by Moraceae and Fabaceae. In the Indochinese silvered langur samples, 27 families were recorded with Moraceae found in all samples, followed by Phyllanthaceae and Fabaceae. A total of 18 families was identified in the Tonkin snub-nosed monkey samples, with Annonaceae being the most dominant plant family, followed by Lauraceae and Sapotaceae.

Diet metabarcoding also identified new diet plant records which were not previously documented by field observations. For black-shanked doucs, I added 18 families, 15 genera and 13 species to their dietary profile. For Indochinese silvered langurs and Tonkin snub-nosed monkeys, nine families, 18 genera, and 14 species, and three families, five genera, and three species were added as new diet records respectively.

The dietary profiles between the black-shanked douc populations as retrieved from metabarcoding were quite different. There was very little overlap in the food plants identified between the four populations with only six unique sequences/MOTUs common to all. The top diet families among the douc populations were also different from each other, with only Annonaceae and Moraceae being common to the populations in Ta Kou Nature Reserve and Hon Heo Mountain.

Based on metabarcoding and previous field observations, Moraceae was the dominant family in the diet of the Indochinese silvered langurs in Kien Luong Karst Area in Vietnam, demonstrating the importance of this plant family in their diet. However, there was otherwise little overlap in the food species identified through diet metabarcoding and field observations as only two species, *Gymnanthera oblonga* (Apocynaceae) and *Leucoena leucocephala* (Fabaceae) overlapped as food plants for the langurs. Even though Poaceae was the dominant family based on the number of taxa recorded within the Karst Area, no sequences corresponding to this family was detected in the Indochinese silvered langur fecal samples. This observation could be explained by the lack of reference barcodes as more than half of the taxa of Poaceae recorded from the Karst Area were not represented in GenBank, or that Poaceae did not contribute to the diet of the langurs during the time of collection of fecal samples.

In Khau Ca Area where the fecal samples of Tonkin snub-nosed monkeys were collected, *Polyalthia* and Annonaceae were the dominant genus and family respectively based

on abundance. Interestingly, the most dominant plant sequence found in all samples of the Tonkin snub-nosed monkeys was *Polyalthia* of the Annonaceae family, demonstrating a positive selection of the most abundant plants within their habitat.

8.3. Conservation Implications and Recommendations for Future Studies

The patterns of mitochondrial HV-I genetic diversity of the black-shanked doucs were indicative of a lack of recent gene flow between the populations examined, as represented by unique haplotypes in each of the four populations, i.e. no shared haplotypes. These populations are completely isolated from each other (77 – 232 km between any two sites). Population-exclusive mitochondrial haplotypes have also been documented in the critically endangered northern muriquis (*Brachyteles hypoxanthus*) of the Atlantic Forest, in which almost all (19 of 23) haplotypes were exclusively found in one of eight populations examined, and four populations harbored 22 of 23 haplotypes (Chaves *et al.* 2011). The authors recommended that these four populations be considered as discrete units for conservation. For the black-shanked doucs, more fecal samples should be collected from the study sites in Ta Kou, Cat Tien, Nui Chua and Hon Heo, and also from other sites where significant populations of the species are found, such as Bu Gia Map National Park in Vietnam and Seima Biodiversity Conservation Area in Cambodia. If these populations are indeed isolated repositories of the species' genetic diversity, then they should be regarded as management units, especially under the condition of further shrinking distribution and increasing isolation (see Moritz 1994). Of special note is Hon Heo Mountain – which is not a protected area. If there is reason to believe that the population of black-shanked doucs at Hon Heo is viable, it would be worth prioritizing resources and activities toward their protection.

The Indochinese silvered langur is a flagship species of Kien Luong Karst Area in Kien Giang Province, home to the largest population in Vietnam (Hoang Minh Duc *et al.* 2010c; Tran Van Bang *et al.* submitted). It is estimated that there are more than 250

individuals left in the wild here, of which 40% are found within Khoe La hill. Significant habitat losses coupled with fragmentation owing to limestone quarrying activities are restricting population movement and decreasing food resources in Khoe La, whose population is the only one containing haplotypes that can also be found in the other three hill sites in the Karst Area; i.e. local extirpation of the Khoe La population will lead to the isolation of the genetic haplotypes within the Karst Area given that each of the other three populations does not share haplotypes. Immediate action to move the Indochinese silvered langurs out of Khoe La into the other sites is necessary. Given that active translocation is often risky (especially for unhabituated and arboreal Indochinese silvered langurs inhabiting karst hills) and may result in the death of the animals during capture and transport, constructing rope ladders between fragmented habitats can be explored to restore wildlife movement (e.g. Teixeira *et al.* 2013 for brown howler monkey, *Alouatta guariba clamitans* in Porto Alegre, Brazil) and reestablish genetic connectivity between the populations. Once artificial green corridors are created, the langurs would have higher breeding opportunities, greater resource availability, and hence an elevated probability of population survival in the future.

Fifty km west of the Kien Luong coast is Phu Quoc Island, home to a small population of at least 54 individuals of Indochinese silvered langurs (Tran Van Bang *et al.* submitted). Phu Quoc Island is believed to have been separated from mainland since the most recent glacial period (Fontaine & Workman 1978; Rainboth 1996). Indochinese silvered langur populations could have "island-hopped" to Phu Quoc Island in the past 12,000 years via smaller islands between Phu Quoc and the geographically closer mainland Cambodia. It would be interesting to examine the genetic variability of the Phu Quoc population to provide an overview of the genetic diversity of the species in Vietnam and to investigate if there were recent gene flow between them.

The Tonkin snub-nosed monkey population in Khau Ca Area is arguably the most important population for the survival of the species with by far the largest population (Le Khac Quyet *et al.* 2015). This population has also been reproducing, with infants being observed for the last 10 years (Le Khac Quyet, personal communication). Despite an apparent population growth over the years, the absence of any variability for the HV-I region and the entire mitochondrial genome is a major concern. Low genetic variability of the Tonkin snub-nosed monkeys in Khau Ca Area may be a result of widespread deforestation and intensive hunting in recent decades which severely fragmented the habitat (Nadler 2014), resulting in restricted gene flow (the closest other population in Tung Vai Forest is 35 km away). This result highlights the immediate need for a comprehensive assessment of the genetic diversity in all remaining populations of Tonkin snub-nosed monkeys especially Tung Vai population of ca. 20 individuals based on both mitochondrial and nuclear markers. The latter needs to be developed for this species.

Diet metabarcoding is an efficient tool to identify food species and characterize species compositions in fecal samples at the population level within a short period of time (taking into account sample collection, DNA extraction, amplification, sequencing, and analysis). The downstream identification of sequences retrieved from fecal samples, however, is limited by the adequacy of the sequence database. In Chapters 5-7, I quantified the level and accuracy of plant identifications to each of the sequences detected in the fecal samples and found that identifications varied depending on which database was used as the taxonomic reference, i.e. there is no one database (global or local) at the moment that is the best reference database, given that not all known plant species found within the habitats of the primates have representative DNA barcodes in global databases such as GenBank. For all the sites examined in this dissertation, I found that 60-74% of the plant taxa and 15-28% of the plant genera known in the study sites were not represented in GenBank by their P6 loop of

chloroplast trnL sequence, which is the most commonly used barcode for plant identification. Therefore, in order for diet metabarcoding to be a valuable tool to supplement field observational studies on feeding ecology of primates, large-scale barcoding of plant taxa within their habitats needs to be carried out so as to augment the current database. This would entail setting up botanical plots within the habitats, identifying the angiosperms present, sampling leaves of identified taxa, extracting DNA, amplifying and sequencing chloroplast 'barcoding' regions (such as trnL, rbcL, and matK), depositing the sequences into global databases such as GenBank, and vouchering the specimens. The inclusion of more than one plant barcode would help refine the taxonomic identifications of closely related species. In addition to plant barcodes, future research can also explore the sequencing of invertebrates, such as arthropods, found in the diets of colobine primates using mitochondrial cytochrome *c* oxidase subunit I (COI).

A number of colobine primates have been observed to engage in geophagy (see Krishnamani & Mahaney 2000). In Cambodia, Rawson and Luu Tuong Bach (2011) reported that red-shanked doucs and Indochinese silvered langurs regularly descend to the ground to eat soil from salt licks, which is likely a behavior to counter the toxicity in the leaves consumed and/or to supplement their diet with minerals. While diet metabarcoding is unable to detect this behavior, visual examination of the interior of their fecal material would enable researchers to detect soil if consumed in significant amounts; armadillos (family Dasypodidae) swallow large amounts of soil in the processing of eating termites and ants, and in turn large quantities of soil particles were detected in their feces (Vaz *et al.* 2012).

8.4. Conclusions

This dissertation has explored the use of genetic methods to complement traditional field methods to study population viability and feeding ecology of primates, especially highly threatened species so as to help with their conservation. In order to identify appropriate and

sound conservation actions specific to each population and species, information such as genetic variability and dietary profiles is essential. My research has generated the first information on mitochondrial genetic variability of three species of colobine primates in Vietnam and has helped identify populations requiring priority conservation actions and key areas requiring support (i.e. financial, manpower, and/or research). In this way, resources available can be better managed for targeted outcomes. This study has also assessed the efficacy and drawbacks of diet metabarcoding on primate fecal samples. I was able to perform rapid plant diet analysis to generate population-level dietary profiles of the primates. This information is useful for identifying important food plants and understanding diet choices in relation to habitat flora diversity. However, diet metabarcoding has low taxonomic resolution in plant identifications when sequence databases do not have sufficient coverage of the flora diversity in the primate habitats. I have highlighted that an important step toward a large-scale and comprehensive examination of primate diet at the individual and population levels over an extended period of time should begin with the barcoding of plant taxa within their habitats, followed by exploring the use of both field observations and diet metabarcoding. I hope that this dissertation helps identify priority actions for the successful conservation of the colobine primates in Vietnam through a better understanding of their genetic variability and diet choices. Ultimately, this study builds on the growing work into the research and conservation of colobine primates and would be useful if it encourages further research into colobine primates in Vietnam and in the region.

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APPENDICES

Appendix A. List of plant taxa without corresponding trnL sequences in GenBank. These taxa were not represented in my local databases.

Cat Tien	Ta Kou		Kien Gang		Khau Ca	
194 Genera	**93 Genera**	**441 Species**	**90 Genera**	**569 Species**	**52 Genera**	**318 Species**
Acriopsis	*Aegynetia*	*Abelmoschus crinitis*	*Aclisia*	*Abelmoschus moschatus*	*Acanthopanax*	*Abelmoschus moschatus*
Actephila	*Ancistrocladalus*	*Abelmoschus moschatus*	*Acrostichum*	*Abutilon indicum*	*Adenosma*	*Acampe rigida*
Actinodaphne	*Antrophyum*	*Abutilon indicum*	*Adenostemma*	*Acacia farnesiana*	*Aglaomorpha*	*Acanthopanax gracilistylus*
Aglaomorpha	*Asplenium*	*Acacia harmandiana*	*Aglaodora*	*Acacia magium*	*Amischotolype*	*Achyranthes bidentata*
Agrostophylium	*Azardiracta*	*Acalypha acmophylla*	*Aglaomorpha*	*Aclisia secundiflora*	*Aporosa*	*Adenosma caeruleum*
Alcasia	*Baccaurea*	*Adenia parviflora*	*Ancistrocladus*	*Acorus verus*	*Asplenium*	*Adiantum caudatum*
Allomorphia	*Balanophora*	*Adenia pierrei*	*Aporosa*	*Acrostichum aureum*	*Belamcanda*	*Aeschynanthus garrettii*
Alseodaphne	*Bolbitis*	*Adenia viridiflora*	*Asplenium*	*Acrostichum speciosum*	*Calcareoboea*	*Aeschynanthus longicaulis*
Alysicarpu	*Brownlowia*	*Adiantum caudatum*	*Azima*	*Adenia parviflora*	*Ceratostylis*	*Aglaomorpha coronans*
Amischolotype	*Calycopteris*	*Aegynetia indica*	*Baccaurea*	*Adenia viridiflora*	*Chirita*	*Aglaonema siamense*
Amoora	*Carmone*	*Aeschynomene americana*	*Balakata*	*Adenostemma lavenia*	*Chukrasia*	*Aglaonema simplex*
Amphicarpa	*Cassytha*	*Afzelia xylocarpa*	*Balanophora*	*Aerva sanguinolenta*	*Cibotium*	*Aidia cochinchinensis*
Ancistrocladus	*Castanapsis*	*Aganosma acuminata*	*Basella*	*Aeschynomene americana*	*Cirrhopetalum*	*Aidia oxyodonta*
Anogeissus	*Cenchorus*	*Aglaia pleuropteris*	*Belamcanda*	*Aeschynomene aspera*	*Cleistocalyx*	*Aidia pycnantha*
Anomianthus	*Cenolophon*	*Aglaia poilanei*	*Boerhavia*	*Afzelia xylocarpa*	*Crepidomanes*	*Alangium chinense*
Antrophyum	*Centhothera*	*Aglaonema ovatum*	*Cassytha*	*Aglaia cucullata*	*Ctenopteris*	*Alangium kurzii*
Aporosa	*Cheilanthes*	*Aglaonema tenuipes*	*Ceratopteris*	*Aglaodora griffithii*	*Cyclosorus*	*Allophylus caudatus*
Arnicratca	*Colona*	*Agrostophyllum khasianum*	*Chasallia*	*Aglaomorpha coronans*	*Cyrtosia*	*Alstonia angustifolia*
Aronychia	*Cratoxylon*	*Agrostophyllum panicaule*	*Chirita*	*Aidia oxyodonta*	*Decaspermum*	*Alyxia siamensis*
Artanema	*Cryptophragmium*	*Albizia vialenea*	*Christella*	*Ailanthus triphysa*	*Dichroa*	*Amaranthus caudatus*
Ascocentron	*Ctenitopsis*	*Allophylus brachypetalus*	*Codariocalyx*	*Albizia odoratissima*	*Diplazium*	*Amischotolype mollissima*
Asplenium	*Davallia*	*Alstonia angustifolia*	*Cratoxylum*	*Alchornea rugosa*	*Drynaria*	*Amorphophallus tonkinensis*
Aternanthera	*Diplazium*	*Ampelocissus martini*	*Cyclosorus*	*Alpinia conchigera*	*Duchesnea*	*Ampelocalamus patellaris*
Baccaurea	*Drynaria*	*Ancistrocladalus wallichii*	*Decaschistia*	*Alpinia oxymitra*	*Eleuthrine*	*Ampelopsis cantoniensis*

Benincasia
Bergia
Biermannia
Biophitum
Blatus
Cacanga
Calycopteris
Campestigma
Cardiopterris
Cassytha
Centrosema
Cephaelis
Cephalomanes
Cephlantheropsis
Ceratophylum
Ceratopteris
Champerela
Cheilanthes
Chirita
Chrysalidocarpus
Cibotium
Clathea
Cleistocalyx
Colona
Cornopteris
Cratoxylon
Crypteronia
Cryptosperma
Cyathocalys
Cyclacanthus
Cyclopeltis
Egenolfia
Epipogeum
Epipremmum
Eragostis
Erycoma
Flickingeria
Fomes
Garcinia
Gastrochilus
Glinus
Gnetum
Gymnopelatum
Haldina
Helicteres
Hemmigraphis
Homalonema
Irvingia
Justica
Lindenia
Lindsaea
Linociera
Marsilea
Mecopus
Melientha
Memecylon
Microsorum
Milletia
Mukia
Munronia
Murdannia
Musaenda
Ancistrocladus cochinchinensis
Ancistrocladus tectorius
Anisoptera costata
Antidesma cochinchinensis
Antidesma ghaesembilla
Antidesma japonica
Antidesma poilanei
Archidendron balansae
Archidendron occultatum
Ardisia amherstiana
Ardisia dinhensis
Arenga pinnata
Argyreia acuta
Argyreia mekongensis
Argyreia pierreana
Artabotrys intermedius
Artocarpus chapasla
Asplenium hainanense
Asplenium nidus
Asplenium oldhami
Azardiracta indica
Baccaurea ramiflora
Balanophora fungosa
Balanophora latisepala
Bambusa flexuosa
Bambusa procera
Bambusa schzostachyoides
Bauhinia bracteata
Bauhinia malabarica
Bauhinia saigonensis
Bauhinia viridescens
Deeringia
Dichroa
Dicranopteris
Diplazium
Donella
Drynaria
Eichhornia
Epiprinus
Erianthus
Erytrina
Floscopa
Garcinia
Glinus
Gomphia
Halophila
Heilianthus
Helicteres
Hydrocera
Hymenocardia
Irvingia
Juncellus
Lasia
Alstonia macrophylla
Alstonia spathulata
Alternanthera sessilis
Amaranthus lividus
Amomum thyrsoideum
Ampelocissus humulifolia
Ancistrocladus cochinchinensis
Ancistrocladus tectorius
Anisoptera costata
Antidesma acidum
Antidesma ghaesembilla
Antidesma montanum
Aporosa dioica
Aporosa planchoniana
Aquilaria crassna
Araucaria columnaris
Archidendron clypearia
Archidendron quocense
Ardisia expansa
Ardisia macrosepala
Arenga pinnata
Argusia argentea
Argyreia mekongensis
Aristolochia indica
Artabotrys intermedius
Artocarpus communis
Asplenium affine
Asplenium confusum
Asplenium longissimum
Asplenium nidus
Atalantia roxburghiana
Ellipanthus
Erythropalum
Garcinia
Gnetum
Graptophyllum
Homonoia
Illigera
Iodes
Ixeris
Mahonia
Manglietia
Mariscus
Melientha
Miliusia
Nephrolepis
Oxyspora
Phoebe
Pyrrosia
Radermacheta
Selaginella
Sinosideroxylon
Stichorkis
Tectaria
Tradescentia
Tupistra
Urena
Ventilago
Xanthium
Angiopteris yunnanensis
Aphanamixis grandifolia
Aporosa sphaerosperma
Aralia armata
Archidendron ellipticum
Archidendron occultatum
Archidendron tonkinense
Ardisia silvestris
Arenga pinnata
Argyreia capitata
Arisaema balansae
Arisaema erubescens
Aspidistra tonkinensis
Aspidistra typica
Asplenium nidus
Asplenium saxicola
Asplenium tenuifolium
Bauhinia bracteata
Bauhinia oxysepala
Begonia balansaeana
Begonia cavalerei
Begonia rubrovenia
Begonia tonkinensis
Belamcanda chinensis
Bennettiodendron cordatum
Blechnum orientale
Bulbophyllum hymenanthum
Bulbophyllum longibrachiatum
Calamus platyacanthus
Calcareoboea coccinea
Callicarpa bodinieri

Cyclosorus
Daemonorops
Dasymaschlon
Dehaasia
Desosdium
Deutzianthus
Dicranopteris
Dictyospermum
Didmosperma
Diodia
Diphiectria
Diplobryum
Donax
Donella
Dracuntomelon
Drynaria
Dunbaria
Erianthus
Erythropalum
Erythrorchis
Fibrauria
Fissitigma
Flickingeria
Floscopa
Garcinia
Garruga
Gastrochilus
Gleichenia
Glenniea
Glossocarya
Gmelia
Nephrolepis
Nosema
Notholaena
Oroxylon
Osbeckia
Parinari
Pericampilus
Phoebe
Platycerium
Pollia
Pseuderanthermum
Pseudodracuntium
Psiloesthes
Pteris
Pterocymbium
Pyrrosia
Rauwenhoffia
Rhodomyrtus
Rhopalephora
Salicia
Sauporus
Sebastiana
Securinega
Selaginella
Sphenodesme
Staurochilus
Stenoclena
Tectaria
Thismia
Thysanolena
Tropidia
Begonia lecomtei
Biophytum sensitivum
Blainvillea acmella
Boea microcarpa
Boesenbergia rotunda
Bolbitis crispatula
Bombax albidum
Breynia diversifolia
Breynia vitis-idaea
Bridelia harmandii
Bridelia monoica
Brownlowia denysiana
Buchanania lucida
Burmannia subcoelestis
Caesalpinia hymenocarpa
Calamus balaneanus
Calamus pseudoscutellaris
Calamus tetradaetylus
Calanthe cardioglossa
Callicarpa erioclona
Calophyllum dryobalanoides
Calophyllum touranensis
Calycopteris floribunda
Cananga latifolia
Canarium subulatum
Canthium dicoccum
Canthium parvifolium
Capparis henryi
Carallia lancefolia
Carallia suffruticosa
Carmone microphylla
Avicennia officinalis
Avicennia rumphiana
Azima sarmentosa
Baccaurea ramiflora
Balakata baccata
Balanophora indica
Barringtonia acutangula
Barringtonia micrantha
Basella rubra
Bauhinia bassacensis
Bauhinia bracteata
Bauhinia malabarica
Begonia harmandii
Begonia rupicola
Belamcanda chinensis
Biophytum sensitivum
Blachia andamanica
Blechnum indicum
Boerhavia diffusa
Bombax anceps
Brachiaria eruciformis
Brachiaria mutica
Breynia fruticosa
Breynia vitisidaea
Bridelia monoica
Bridelia ovata
Bridelia stipularis
Bruguiera cylindrica
Bruguiera gymnorrhiza
Bruguiera parviflora
Bruguiera sexangula
Callicarpa brevipes
Canthium umbellatum
Carex adrienii
Carex sikokiana
Cassia tora
Cayratia palmata
Celastrus gemnatus
Celtis japonica
Ceratostylis himalaica
Chloranthus elatior
Chukrasia tabularis
Cibotium barometz
Cinnamomum loureirii
Cinnamomum magnificum
Cirrhopetalum delitescens
Clausena heptaphylla
Clausena indica
Cleistocalyx operculatus
Clematis gouriana
Clerodendrum tonkinense
Coelogyne lactea
Commelina diffusa
Connarus cochinchinensis
Cordyline fruticosa
Costus speciosus
Costus tonkinensis
Crepidomanes bipunctatum
Ctenopteris obliquata
Curculigo capitulata
Curculigo gracilis
Cyathula prostrata

Gnetum
Grangca
Greenia
Gymnospetalum
Haldina
Helicteres
Helminthostachys
Hemionotis
Heynia
Homalonema
Homonoia
Humata
Hymenocardia
Hymenopogon
Illigera
Irvingia
Ixonanthes
Kingidium
Laffa
Lasia
Lindsaea
Linociera
Ludwidgia
Macclurodendron
Malleola
Malvastrum
Manglietia
Manikara
Marsilea
Mecopus
Melanorrhoea
Urena
Ventilago
Veronia
Willughbeia
Xanthium
Zanonia
Zollingeria
Caryota sympelata
Cassia mimosoides
Cassia tora
Cassytha filiformis
Castanapsis namdinhensis
Castanopsis argyrophylla
Castanopsis wilsonii
Cenchorus brownii
Centhothera lappacea
Cheilanthes belangeri
Cinnamomum durifolium
Cinnamomum longepetiolatum
Cinnamomum loureiri
Cissus astrotricha
Cissus evrardii
Cissus hexagularis
Cissus modeccoides
Cissus rosea
Claoxylon indicum
Clausena dimidiata
Cleistanthus indochinensis
Clematis henryi
Clematis smilacifolia
Clematis subapelta
Clerodendrum lanessanii
Clerodendrum palmatolobatum
Colona auriculata
Colona thorelii
Combretum deciduum
Combretum punctatum
Connarus cochinchinensis
Bulbostylis puberula
Byttneria aspera
Caesalpinia bonduc
Calamus rudentum
Calamus tetradactylus
Calamus viminalis
Canarium subulatum
Canavalia cathartica
Canavalia maritima
Canthium cochinchinense
Canthium glabrum
Capparis diffusa
Capparis micracantha
Capsicum frutescens
Carallia eugenioides
Carallia lanceaefolia
Carapa mekongensis
Carex indica
Careya sphaerica
Caryota monostachya
Casearia grewiaefolia
Cassytha filiformis
Cayratia triflolia
Celosia argentea
Centratherum intermedium
Ceratopteris thalictroides
Cereus peruvianus
Chaetocarpus castanocarpus
Chamaecrista mimosoides
Chasallia curviflora
Chirita hamosa
Cyclosorus truncatus
Cyrtosia javanica
Decaspermum parviflorum
Dendropanax macrocarpus
Dichroa hirsuta
Didissandra aspera
Dillenia indica
Dillenia turbinata
Diospyros bonii
Diospyros quaesita
Diplazium esculentum
Dischidia tonkinensis
Disporopsis longifolia
Disporum cantoniense
Disporum trabeculatum
Drynaria bonii
Drynaria fortunei
Dryopteris fuscipes
Duchesnea indica
Elaeocarpus griffithii
Elatostema colonieae
Eleuthrine bulbosa
Ellipanthus tomentosus
Eria coronaria
Eria pannea
Erycibe hainanensis
Erythrina variegata
Erythropalum scandens
Etlingera pavieana
Euodia lepta
Euodia meliaefolia

Melochia
Mesua
Metadina
Micromclum
Micropera
Monochoria
Murdannia
Musssaenda
Neruum
Nosema
Ochrocarpus
Oleandra
Onchidanthera
Oraithochilus
Oroxylon
Osbeckia
Ottelia
Palaquium
Parinari
Pentaphragma
Pericampilus
Phalenopsis
Phoebe
Phytocrene
Pinanga
Pithecellbium
Platycerium
Pollia
Pronephrium
Pseudodracuntium
Pteris

Corchorus aestuans
Costus speciosus
Cratoxylon cochinchinensis
Cratoxylon formosum
Crotalaria montana
Cryptophragmium affine
Ctenitopsis colaniae
Curcuma cochinchinensis
Cyclea peltata
Cymbidium aloifolium
Cymbopogon caesius
Cyperus andreanus
Cyperus leucocephalus
Cyrtococcum accrescens
Dalbergia darlacensis
Dalbergia olivieri
Dendrobium crumenatum
Desmodium gangeticum
Desmodium pulchellum
Desmodium thorelii
Desmodium ursinum
Dialium cochinchinensis
Dianella nemorosa
Dicliptera leonotis
Dioscorea kratica
Dioscorea laurifolia
Diospyros barauensis
Diospyros tauranensis
Diplazium crassiusaelum
Diplocyclos palmatus
Dischidia pseudo-bengalensis

Chirita involucrata
Chloranthus elatior
Christella parasitica
Christia pierrei
Cinnamomum longepetiolatum
Cissus modeccoides
Cissus rosea
Cissus triloba
Cleistanthus hirsutulus
Cleistanthus sumatranus
Cleome viscosa
Clerodendrum cochinchinense
Clerodendrum godefroyi
Clerodendrum inerme
Clerodendrum palmatolobatum
Clerodendrum serratum
Clerodendrum wallichii
Clitoria hanceana
Codariocalyx motorius
Coix aquatica
Coix gigantea
Combretum quadrangulare
Combretum tetralophum
Commelina communis
Commelina diffusa
Commelina obliqua
Commelina paludosa
Connarus cochinchinensis
Connarus paniculatus
Corchorus olitorius
Cordia cochinchinensis

Euphorbia thymifolia
Fernandoa brilletii
Fibraurea recisa
Ficus henryi
Ficus hirta
Ficus obscura
Ficus pubigera
Ficus variegata
Fissistigma oldhamii
Garcinia brataeta
Garcinia fagraeoides
Garcinia multiflora
Garcinia tinctoria
Glochidion eriocarpum
Glycosmis cyanocarpa
Glycosmis sinensis
Gnetum montanum
Gomphandra tetrandra
Graptophyllum pictum
Gynura colaniae
Hedyotis capitellata
Heterostemma brownii
Hiptage lucida
Hodgsonia macrocarpa
Homalomena occulta
Homonoia riparia
Hoya balansae
Huperzia hamiltonii
Huperzia phlegmaria
Illicium cambodianum
Illicium macranthum

Pterocymbium
Pyrrosia
Quisqualis
Rauwenhoffia
Rhodomyrtus
Rhopalephora
Robiquetia
Rottboellia
Rotula
Sageraca
Salvini
Samadeda
Sandoricum
Sarcoglyphis
Sauropus
Scaphium
Securinega
Selaginella
Semecapus
Sinosideroxylon
Sphenodesma
Staurochilus
Stenochlaena
Sticherus
Swintoma
Tabernacmontana
Taeniophyllum
Taenitis
Taladiantha
Tarmarindus
Tectaria

Dischidia tonkinensis
Drosera indica
Drynaria bonii
Drynaria fortunei
Drynaria quercifolia
Dysoxylum loureirii
Egenolfia appendiculata
Elaeagnus conferta
Elaeocarpus harmadii
Emilia sonchifolia
Epipogeum roseum
Epipremmum giganteum
Eragostis zeylanica
Erycoma longifolia
Erythrina variegata
Erythroxylum cuneatum
Erythroxylum gracile
Euodia lepta
Eupatorium odoratum
Euphorbia antiquorum
Fernandoa serrata
Ficus capillipes
Ficus chartacea
Ficus curtipes
Ficus depressa
Ficus drupecea
Ficus hirta
Ficus kurzii
Ficus pisocarpa
Ficus racemosa
Ficus rumphii

Cordia subcordata
Costus speciosus
Crateva adansonii
Cratoxylum cochinchinense
Cratoxylum formosum
Cratoxylum pruniflorum
Crinum defixum
Crinum ensifolium
Crotalaria chinensis
Crotalaria montana
Crotalaria quinquefolia
Cryptocoryne ciliata
Cyanotis axillaris
Cyanotis cristata
Cycas lithoralis
Cycas pectinata
Cyclosorus interruptus
Cymbidium aloifolium
Cynanchum stauntonii
Cyperus babakan
Cyperus diffusus
Cyperus digitatus
Cyperus elatus
Cyperus exaltatus
Cyperus halpan
Cyperus imbricatus
Cyperus leucocephalus
Cyperus malaccensis
Cyperus stoloniferus
Cyperus trialatus
Dalbergia candenatensis

Illicium majus
Illicium parvifolium
Illigera cucullata
Impatiens verrucifer
Iodes cirrhosa
Iodes seguini
Iresine herbstii
Ixeris dentata
Ixora diversifolia
Ixora henryi
Ixora umbellata
Jasminum pedunculatum
Jasminum subtripliverve
Justicia curviflora
Laportea violacea
Licuala tonkinensis
Limacia scandens
Lindernia ruellioides
Liparis bootanensis
Liparis mannii
Litsea cubeba
Litsea monopetala
Luisia filiformis
Lycianthes denticulata
Lygodium conforme
Lysimachia chapaensis
Lysionotus serratus
Machilus platycarpa
Maesa balansae
Maesa membranacea
Maesa montana

Telectadium
Tephrisia
Thelypteris
Tithonia
Trias
Trichglottis
Urena
Ventilago
Vittaria
Walsura
Willughberia
Winchia
Xanthoxylum
Zizyphus
Zollingeria

Ficus spathulifolia
Ficus tinctori
Fimbristylis insignis
Flacourtia indica
Flemingia strobilifera
Fomes japonicus
Garcinia benthami
Garcinia merguensis
Garcinia oblongifolia
Garcinia schefferi
Gardenia obtusifolia
Gastrochilus thorelii
Glinus hernarioides
Glinus lotoides
Globba annamensis
Globba cambodgensis
Glochidion sphaerogynum
Glycosmis cymosa
Glycosmis gracilis
Glycosmis ovoides
Glycosmis sapindoides
Gmelina asiatica
Gnetum latifolium funiculare
Gnetum montanum
Gomphostemma grandiflorum
Goniothalamus donnaiensis
Gonocaryum lobbianum
Gouania javanica
Grewia abutilifolia
Grewia astropetala
Grewia hirsuta

Decaschistia parviflora
Deeringia polysperma
Dendrobium aloifolium
Dendrobium crumenatum
Dendrobium polyanthum
Dendrolobium rostratum
Dendrolobium umbellatum
Derris trifoliata
Desmodium styracifolium
Desmodium triflorum
Dichroa febrifuga
Dicranopteris linearis
Digitaria petelotii
Digitaria setigera
Dillenia indica
Dillenia ovata
Dioscorea cambodiana
Dioscorea trinervia
Diospyros variegata
Diplazium esculentum
Dipterocarpus costatus
Dipterocarpus dyeri
Dipterocarpus intricatus
Dischidia major
Dischidia nummularia
Donella lanceolata
Dregea volubilis
Drynaria bonii
Drynaria quercifolia
Dysoxylum cochinchinense
Dysoxylum rubrocostatum

Maesa perlarius
Mahonia nepalensis
Manglietia conifera
Manglietia insignis
Mariscus umbellatus
Marsdenia tinctoria
Marsdenia tonkinensis
Melientha suavis
Melodinus tenuicaudatus
Michelia balansae
Michelia mediocris
Microdesmis casearіaefolia
Miliusia sinensis
Millettia pulchra
Mimusops elengi
Mussaenda cambodiana
Naravelia laurifolia
Neolitsea buisanensis
Nephrolepis cordifolia
Nervilia fordii
Ophiopogon longifolius
Ophiopogon reptans
Ophiorrhiza amplifolia
Ophiorrhiza baviensis
Ophiorrhiza japonica
Oreocnide rubescens
Oxalis corymbosa
Oxyspora paniculata
Pavetta translucens
Pegia sarmentosa
Phaius indochinensis

Grewia oligandra
Grewia paniculata
Grewia polygama
Grewia tomentosa
Gymnopelatum cochinchinensis
Gynura procumbens
Gynura pseudochina
Haldina cordifolia
Hedyotis biflora
Hedyotis scoparia
Hedyotis simplicissima
Helicteres angustifolia
Helicteres hirsuta
Helicteres lanceolata
Helicteres viscida
Heliotropium indicum
Hemmigraphis glaucescens
Hibiscus squamosus
Hibiscus surattensis
Homalonema occulta
Hopea odorata
Hoya fusca
Hoya macrophylla
Hoya minima
Hunteria zeylanica
Ipomoea involucrata
Irvingia malayana
Ischaemum barbatum
Ischaemum rugosum
Ixora cuneifolia
Ixora eugenoides
Eichhornia crassipes
Elaeocarpus griffithii
Elaeocarpus hygrophilus
Elaeocarpus tectorius
Eleocharis ochrostachys
Eleocharis retroflexa
Eleocharis spiralis
Elsholtzia blanda
Embelia ribes
Emilia sonchifolia
Entada rheedii
Enydra fluctuans
Epiprinus siletianus
Epithema brunonis
Eragrostis diplachnoides
Eragrostis malayana
Erechtites valerianaefolia
Eriachne chinensis
Erianthus arundinaceus
Eriocaulon basscense
Eriocaulon intermedium
Eriocaulon nautiliforme
Eriochloa procera
Erytrina variegata
Eulalia leschenaultiana
Eulophia graminea
Euodia callophylla
Euodia lepta
Euonymus cochinchinensis
Eupatorium odoratum
Euphorbia antiquorum
Phlogacanthus annamensis
Phoebe kunstleri
Pholidota leveilleana
Pholidota rubra
Pholidota yunnanensis
Photinia arguta
Phragmites maximus
Phrynium dispermum
Phytolacca acinosa
Pilea notata
Pinus kwangtungensis
Piper bonii
Piper cambodiana
Piper carnibracteum
Piper cubeba
Piper laosanum
Piper lolot
Piper longum
Piper saxicola
Pistacia weinmannnifolia
Pittosporum balansae
Podochilus khasianus
Polyalthia cerasoides
Polyalthia nemoralis
Polygala luteo-alba
Polygala wattersii
Polygonatum punctatum
Polygonum caespitosum
Polygonum chinensis
Pomatocalpa armigerum
Porana racemosa

Ixora laotica
Ixora umbellata
Jasminum adenophyllum
Jasminum subtriplinerve
Justica gendarussa
Justica neesiana
Kaempferia galanga
Kaempferia harmandiana
Knema erratica
Knema pierrei
Lagerstroemia calyculata
Lagerstroemia duperreana
Lagerstroemia ovalifolia
Lasianthus annamicus
Lasianthus chinensis
Lasianthus cupreus
Lasianthus wallichii
Leea crispa
Lindsaea ensifolia
Lindsaea odorata
Lindsaea walkerae
Linociera sangda
Lithocarpus rouletii
Lithocarpus salbulicolus
Litsea cambodiana
Luisia discolor
Luvunga scandens
Lygodium auriculatum
Lygodium conforme
Lygodium digitatum
Lygodium microstachyum
Euphorbia atoto
Euphorbia thymifolia
Eurya japonica
Eurycoma longifolia
Excoecaria indica
Ficus auriculata
Ficus callosa
Ficus fulva
Ficus heterophylla
Ficus hirta
Ficus nervosa
Ficus racemosa
Ficus rumphii
Ficus talbotii
Fimbristylis acuminata
Fimbristylis disticha
Fimbristylis fuscoides
Fimbristylis pauciflora
Fimbristylis sericea
Finlaysonia obovata
Flemingia strobilifera
Floscopa scandens
Flueggea virosa
Garcinia mangostana
Garcinia xanthochymus
Glinus lotoides
Glinus oppositifolius
Globba parva
Globba schomburgkii
Glochidion hirsutum
Glochidion littorale
Pottsia grandiflora
Pratia nummularias
Premna fulva
Pseuderanthemum poilanei
Psychotria balansae
Psychotria bonii
Psychotria rubra
Pterospermum diversifolium
Pyrrosia lanceolata
Pyrrosia lingua
Radermacheta sinica
Randia spinora
Renanthera coccinea
Rhododendron emarginatum
Rhododendron saxicolum
Rhododendron sororium
Rubus alcaefolius
Rubus cochinchinensis
Rubus leucanthus
Rubus ovatus
Rubus tonkinensis
Salvia sonchifolia
Sapium rotundifolium
Saurauia fasciculata
Saurauia roxburghii
Saurauia tristyla
Schefflera palmiformis
Schefflera pes-avis
Scleria terrestris
Selaginella delicatula
Selaginella intermedia

Mallotus clellandii
Mallotus eberhardtii
Mallotus glabriusculus
Mangifera dongnaiensis
Mangifera duperreana
Markhamia stipulata
Marsilea quadrifolia
Mecopus nidulans
Melientha suavis
Memecylon acuminatum
Memecylon caeruleum
Memecylon edule
Memecylon octocostum
Micromelum minitum
Microsorum punctatum
Milletia brandisiana
Milletia diptera
Mimosa diplotricha
Morinda tomentosa
Mucuna brevipes
Mucuna interprupta
Mukia maderaspatana
Munronia sinica
Murdannia edulis
Murdannia giganteum
Murdannia nudiflorum
Musaenda hoaensis
Mussaenda dehiscens
Myrioneuron faberii
Nephrolepis radicans
Nervilia crispata
Glochidion sphaerogynum
Glochidion zeylanicum
Glycosmis ovoidea
Gmelina asiatica
Gomphia serrata
Goniothalamus gabriacianus
Gouania javanica
Grewia annamica
Grewia callophylla
Grewia eriocarpa
Grewia paniculata
Guioa pleuropteris
Gymnopetalum cochinchinense
Gynema yunnanense
Gynochthodes proboscidea
Gynura procumbens
Halophila beccarii
Hedyotis biflora
Hedyotis corymbosa
Hedyotis ovatifolia
Hedyotis tenelliflora
Hedyotis vestita
Heilianthus annuus
Heliconia bihai
Heliconia psittacorum
Helicteres angustifolia
Helicteres isora
Heliotropium indicum
Heritiera macrophylla
Hibiscus sabdariffa
Hibiscus tiliaceus
Setaria barbata
Sida rhombifolia
Sinosideroxylon pedunculatum
Sinosideroxylon wrightianum
Smilax bracteata
Smilax corbularia
Smilax glabra
Smilax menispermoidea
Smilax prolifera
Solanum erianthum
Solanum ferox
Spiranthes acmella
Stephania rotunda
Sterculia hymenocalyx
Sterculia nobllis
Stichorkis distans
Streptocaulon juventas
Strychnos ignatii
Styrax serrulatus
Syzygium jambos
Tabernaemontana balansae
Tabernaemontana bufalina
Taxus chinensis
Tectaria decurrens
Tetrastigma macrocrymbosum
Tetrastigma oliviforme
Thladiantha siamensis
Thysanolaena maxima
Tradescentia zebrina
Trichosanthes baviensis
Tupistra hainnanensis

Nervilia prainiana
Neuropeltis racemosa
Nosema capitatum
Notholaena velutina
Olax scandens
Operculina petaloides
Ophrestia pinnata
Ormosia semicastrata
Oroxylon indicum
Oxyceros vidalii
Pandanus tonkinensis
Panicum luzonense
Parinari annamensis
Pavetta nervosa
Pavetta trachyphylla
Peltophorum dasyrrachis
Pennisetum polystachyon
Peperomia parcilia
Peperomia pellucida
Pericampilus glaucus
Phlogacanthus turgidus
Phoebe lanceolata
Phoebe pallida
Phoebe paniculata
Phoebe tavayana
Piper brevicaule
Piper chaudoccanum
Platycerium grande
Pollia thyrsiflora
Polyalthia intermedia
Polyalthia littoralis
Hiptage candicans
Hiptage triacantha
Homalomena occulta
Hopea ferrea
Hopea odorata
Horsfieldia irya
Horsfieldia thorelii
Hydnocarpus anthelminthica
Hydnocarpus ilicifolia
Hydnophytum formicarum
Hydrocera triflora
Hymenachne acutigluma
Hymenocardia punctata
Hyptis rhomboidea
Ilex cochinchinensis
Indigofera galegoides
Indigofera spicata
Ipomoea stolonifera
Irvingia malayana
Isachne miliacea
Ischaemum barbatum
Ischaemum rugosum
Ixora flavescens
Juncellus alopecuroides
Juncellus limosus
Kaempferia galanga
Kalanchoe crenata
Kalanchoe laciniata
Kalanchoe pinnata
Kandelia candel
Khaya senegalensis
Typhonium flagelliforme
Urena lobata
Vaccinium delavayi
Vaccinium dunalianum
Vaccinium sprengelii
Vanilla annamica
Ventilago leiocarpa
Viburnum lutescens
Wedelia chinensis
Xanthium strumarium
Youngia tenuifolia
Zingiber zerumbet
Zippelia begoniaefolia
Ziziphus oenoplia

Polyalthia luensis
Polyalthia sessiliflora
Polygala malesiana
Pseuderanthermum poilanei
Pseudodracuntium anomalum
Psiloesthes elongata
Psychotria adenophylla
Pteris biaurita
Pteris decrescens
Pteris ensiformis
Pteris venusta
Pterocymbium tinctorium
Pterospermum diversifolium
Pyrrosia nummularia
Pyrrosia piloselloides
Quercus austro-cochinchinensis
Radermachera hainanensis
Randia dasycarpa
Randia fasciculata
Randia uliginosa
Raphistemma pulchelum
Rauwenhoffia siamensis
Rhodomyrtus tomentosa
Rhopalephora scaberrima
Sauporus androgynus
Sauporus assimitis
Sauporus bacciformis
Sauporus poilanei
Schefflera eliptica
Scindapsus annamicus
Sebastiana chamaelea
Lagerstroemia duperreana
Laportea interrupta
Lasia spinosa
Lasianthus verticillatus
Lemna minor
Lemna perpusilla
Leonurus japonicus
Leptochloa chinensis
Leptochloa filiformis
Licuala spinosa
Limnocharis flava
Limnophila chinensis
Limnophila heterophylla
Lindernia anagallis
Lindernia antipoda
Lindernia crustacea
Lindernia viscosa
Lithocarpus cambodiensis
Lithocarpus concentricus
Litsea cubeba
Litsea glutinosa
Litsea monopetala
Litsea umbellata
Livistonia saribus
Ludwigia adscendens
Ludwigia octovalvis
Lumnitzera littorea
Lumnitzera racemosa
Luvunga scandens
Lygodium conforme
Lygodium micotahyum

Securinega spirei
Securinega virosa
Selaginella delicatula
Shorea falcata
Shorea siamensis
Sida rhombifolia
Smilax corbularia
Smilax ovalifolia
Smilax verticalis
Solanum ferox
Sphenodesme pentandra
Staurochilus fascinata
Staurogyne malaccensis
Stemona cochinchinensis
Stemona pierrei
Stenoclena palustris
Stephania pierrei
Stephania rotunda
Sterculia pierrei
Sterculia thorelii
Stereospermum colais
Stereospermum cylindricum
Stixis balansae
Streblus ilicifolia
Streptocaulon griffthii
Streptocaulon horsfieldii
Streptocaulon juventas
Streptocaulon kleinii
Streptocaulon wallichii
Strychnos angustiflora
Strychnos axillaris

Maclura thorelii
Maesa perlarius
Mallotus anisopodus
Mallotus cuneatus
Mallotus eberhardtii
Mallotus glabriusculus
Mallotus oblongifolius
Mallotus philippinensis
Mallotus ustulatus
Mangifera minutifolia
Manilkara hexandra
Manilkara kauki
Margaritaria indica
Mariscus compactus
Mariscus javanicus
Markhamia stipulata
Marsilea quadrifolia
Melaleuca cajuputi
Melastoma osbeckioides
Melastoma saigonense
Melientha suavis
Melochia corchorifolia
Memecylon edule
Memecylon fruticosum
Merremia tuberosa
Michelia alba
Microlepia speluncae
Millettia pubinervis
Mimusops elengi
Monochoria hastata
Monochoria vaginalis

Syzygium boisianum
Syzygium glomerulatum
Syzygium hancei
Syzygium levinii
Syzygium lineatum
Syzygium oblatum
Syzygium petelotii
Syzygium pierrei
Syzygium stictanthum
Tectaria brachiata
Tectaria simonsii
Terminalia bellirica
Tetracera indica
Tetracera scandens
Tetrastigma quadridens
Thelasis pygmea
Thespesia lampas
Thismia javanica
Thysanolena maxima
Tinospora cordifolia
Tinospora crispa
Torenia poilanei
Toxocarpus wightianus
Trichoglottis retusa
Trichosanthes pedata
Trigonostemon murtonii
Triumfetta grandidens
Tropidia curculigoides
Turpinia montana
Uncaria sessilifructus
Uraria crinita
Morindopsis capillaris
Mukia maderaspatana
Murdannia vaginata
Musa nana
Musa paradisiaca
Myrsine cochinchinensis
Myrsine linearis
Nymphoides indicum
Nypa fructicans
Oberonia gammiei
Oenanthe javanica
Oryza minuta
Oxystema esculentum
Paederia consimilis
Pandanus humilis
Pantadenia adenanthera
Paraboea cochinchinensis
Parinari annamensis
Peltophorum dasyrrhachis
Peperomia pellucida
Philydrum lanuginosum
Phoebe cuneata
Phoenix paludosa
Phragmites karka
Phragmites vallatoria
Phyllanthus geoffroyi
Phyllanthus reticulatus
Physalis angulata
Pinanga sylvestris
Pluchea pteropoda
Polycarpaea arenaria

Urena lobata
Veronia subacaulis
Vitex canescens
Vitex peduncularis
Vitex pinnata
Wikstroemia androsaemifolia
Willughbeia cochinchinensis
Xanthium inaequilaterum
Zanthoxylum avicennae
Zehneria maysorensis
Ziziphus cambodiana
Ziziphus incurva
Ziziphus oeplia
Zollingeria dongnaiensis

Polycarpon indicum
Polygonum barbatum
Polygonum glabrum
Polygonum hydropiper
Polygonum odoratum
Polygonum persicaria
Polygonum tomentosum
Pouzolzia auriculata
Premna corymbosa
Pseudoraphis brunoniana
Pseudoraphis spinescens
Psilotrichum ferrugineum
Pteris vittata
Pterospermum diversifolium
Pterospermum grewiaefolium
Pycreus globosus
Pycreus polystachyus
Pycreus pumilus
Pyrrosia longifolia
Quassia indica
Quisqualis indica
Randia dasycarpa
Rauvolfia cambodiana
Rauvolfia micrantha
Rhapis cochinchinensis
Rhodamnia dumetorum
Rhodomyrtus tomentosa
Rourea minor
Rubus blepharoneurus
Rubus leucanthus
Ruellia tuberosa

Sacciolepis interrupta
Sacciolepis myuros
Salvinia cucullata
Salvinia natans
Sarcolobus globosus
Sarcostemma acidum
Sauropus androgynus
Sauropus heteroblastus
Sauropus pierrei
Scaphium macropodium
Scindapsus officinalis
Scirpus grossus
Scirpus litoralis
Scirpus mucronatus
Scleria caricina
Scleria ciliaris
Scleria neesii
Scleria poaeformis
Scleria purpurascens
Scleria terrestris
Sclerostachya milroyi
Scolopia macrophylla
Selliguea lateritea
Senna surattensis
Senna tora
Sesbania grandiflora
Sesbania paludosa
Setaria pallide-fusca
Sida rhombifolia
Smilax bracteata
Smilax cambodiana

Smilax ovalifolia
Sphenoclea zeylanica
Spilanthes acmella
Spilanthes oleracea
Spondias cytherea
Sporobolus humilis
Sporopolus virginicus
Staurogyne malaccensis
Stenochlaena palustris
Stephania pierrei
Stephania rotunda
Sterculia rubiginosa
Stereospermum colais
Streptocaulon juventas
Strychnos angustiflora
Strychnos axillaris
Strychnos thorelii
Suregada cicerosperma
Suregada glomerulata
Suregada multiflora
Symplocos laurina
Symplocos lucida
Syzygium cuminii
Syzygium grande
Syzygium lineatum
Syzygium oblatum
Syzygium polyanthum
Tabernaemontana bufalina
Tabernaemontana divaricata
Tabernaemontana pauciflora
Tarenna asiatica

Tarrietia javanica
Tephrosia tinctoria
Terminalia bellirica
Terminalia calamansanai
Terminalia procera
Terminalia triptera
Ternstroemia penangiana
Tetracera scandens
Tetrastigma oliviforme
Tetrastigma strumarium
Thuja orientalis
Thunbergia fragrans
Thunbergia grandiflora
Thysanolaena maxima
Torenia thorelii
Triadica cochinchinensis
Trichosanthes tricuspidata
Trigonostemon quocensis
Trigonostemon reidioides
Trigonostemon rubescens
Triumfetta rotundifolia
Typha javanica
Uncaria acida
Uncaria ovalifolia
Urena lobata
Utricularia bifida
Utricularia punctata
Vallisneria natans
Ventilago cristata
Vernonia cinerea
Vernonia elliptica

		Vetiveria zizanioides *Vigna adenantha* *Vigna luteola* *Vitex canescens* *Vitex pinnata* *Walsura robusta* *Wrightia pubescens* *Xanthium inaequilaterum* *Xantonnea quocensis* *Xylocarpus granatum* *Xylocarpus moluccensis* *Xyris indica* *Xyris pauciflora* *Zanonia maysorensis* *Zehneria indica* *Zingiber gramineum* *Zingiber zerumbet* *Ziziphus oenoplia*	

Appendix B. Renkonen index (RE values) of positive and negative controls and all fecal samples. Fecal samples with RE values less than 0.131 are highlighted red (sample L81), and removed from further analyses.

Species	Sample	Renkonen Index (RE Values)
"Control"	Negative	0.131
	Positive	0.699
Black-shanked douc	5Jun12Sample1	0.760
	6Jun12Sample1	0.801
	6Jun12Sample2	0.838
	6Jun12Sample3	0.883
	7Jun12Sample3	0.856
	L1.1	0.959
	L4	0.946
	L6	0.889
	L7	0.935
	L11.2	0.988
	L12	0.939
	L16	0.975
	L17.1	0.765
	L18.2	0.953
	L19	0.924
	L20	0.942
	L21	0.718
	L22	0.944
	L23	0.954
	L24	0.841
	L25	0.866
	L27.1	0.951
	L28.1	0.969
	L29	0.908
	L30.1	0.914
	L32.1	0.815
	L33	0.965
	L35	0.965
	L41.1	0.921
	L74	0.935
	L75	0.959
	L76	0.972
	L77.1	0.958
	L81	0.039
	L82	0.466
	L83	0.679
	L84	0.936
Indochinese silvered langur	23Sample3	0.923
	23Sample4	0.886
	Sample 6	0.562
	Sample 7	0.590
	Sample 8	0.822
	Sample 9	0.342
	Sample 10	0.585
	Sample 11	0.557
	S1	0.389

	S3	0.917
	S5	0.277
	S6	0.567
	S9	0.917
	S10	0.973
	S11	0.953
	S12	0.921
	S13	0.961
	S14	0.955
	S17	0.963
	S18	0.960
	S19	0.966
	S20	0.968
	S22	0.878
	S25	0.652
	S30	0.965
	S31	0.832
	S38	0.783
	L44	0.847
	L45	0.957
	L50	0.933
	L56	0.955
	L57	0.956
	L58	0.926
	L59	0.946
	L60	0.968
	L61	0.959
	L62	0.963
	L63	0.986
	L65	0.967
	L68	0.941
Tonkin snub-nosed monkey	T1	0.947
	T2	0.990
	T3	0.142
	T4	0.899
	T5	0.929
	T6	0.867
	T7	0.983
	T9	0.954
	T10	0.914
	T11	0.984
	T12	0.906
	T13	0.946
	T14	0.897
	T15	0.947
	T16	0.957
	T17	0.947
	T18	0.934
	T19	0.958
	T20	0.922
	T21	0.971
	T22	0.877
	T24	0.925

T26	0.865
T27	0.889
T28	0.886
T29	0.927
T30	0.960
T31	0.922
T32	0.963
T33	0.941
T34	0.965
T35	0.923
T36	0.982
T37	0.952
T38	0.946
T39	0.669
T40	0.992
T41	0.996
T42	0.949
T43	0.992
T45	0.994
T46	0.971
T47	0.928
T48	0.913
T49	0.926
T50	0.990
T51	0.972
T52	0.982
T53	0.991
T54	0.989
T55	0.991
T56	0.936
T57	0.988
T58	0.994
T59	0.987
T60	0.764
T61	0.990
T62	0.979
T64	0.988
T65	0.964
T66	0.986
T67	0.897
T68	0.966
T69	0.933
T70	0.988
T72	0.994
T73	0.982
T74	0.960
T75	0.989
T78	0.936
T79	0.996
T80	1
T81	0.992
T82	0.992
T83	0.985

T84	0.983
T85	0.983
T86	1
T87	0.994
T88	0.986
T89	0.990
T90	0.999
T92	0.990
T93	0.997
T94	0.995
T95	0.992
T96	0.970
T97	0.962
T98	0.987
T99	0.997
T100	0.973
T102	0.983
T104	0.989
T106	0.986
T107	0.941
T108	0.986
T109	0.937
T114	0.950
T115	0.980
T116	0.989
T117	0.990
T118	0.960
T119	0.985
T120	0.492
T121	0.984
T123	0.993
T127	0.987
T128	0.994
T129	0.983
T130	0.980
T131	0.994
T132	~1 (0.9996)
T133	~1 (0.9998)
T134	~1 (0.9995)
T135	0.995
T137	0.994
T138	0.998
T139	0.980
T140	0.997
T141	0.998
T142	0.998
T143	0.936
T144	0.994
T145	0.989
T146	0.993
T147	0.953
T148	1
T149	0.993

T150	0.998
T152	0.855
T153	0.992
T154	0.982
T156	~1 (0.9997)
T157	0.999
T159	0.930
T160	0.963
T161	0.986
T162	0.971
T163	0.993
T164	0.985
T165	0.979
T166	0.994
T167	0.985
T168	0.941
T169	0.967
T170	0.995
T171	0.996
T172	0.992
T173	0.990
T174	0.991
T176	0.987
T177	0.993
T178	0.986
T179	0.995
T180	0.984
T181	0.957
T182	0.971
T183	0.978
T184	0.989
T185	~1 (0.9999)
T186	0.995
T187	0.989
T188	0.972
T189	0.991
T190	0.944
T191	0.982
T192	0.994
T193	0.989
T194	0.993
T195	0.997
T196	0.987
T198	0.984
T199	0.994
T200	0.998
T201	0.989
T202	0.975
T203	0.993
T204	0.865
T205	0.993
T206	0.989
T207	0.995

T208	0.991
T209	0.984
T211	1
T212	0.986
T213	0.988
T214	0.992
T216	0.989
T218	0.988
T219	0.992
T220	0.987
T222	0.986
T225	0.990
T226	0.996
T227	1
T230	0.995
T233	1
T234	1
T235	0.996
T236	0.994
T237	0.998
T238	0.994
T241	0.995
T242	1
T243	0.996
T244	1
T245	1
T246	0.986
T247	0.991
T248	0.993
T250	0.986
T251	0.983
T252	0.983
T253	0.975
T254	0.977
T255	0.995

Appendix C. Unique sequences identified in BSD samples from all four sites.

Sequence no.	**Sequences**
1	atccggttttctgaaaacaaacaagggttcagaaggcgataataaaaaag
2	atcctgttttctcaaaacaaaacaaaggttcaaaaaacaaaaaaaag
3	atcctgtgttcagaaaacaaggttcagaaagcgagaatcaaaaacagaaaaag
4	atcctgtgttcagcaaacaaggttcagaaagcgagaatcaaaaaaag
5	atccttttttctcaaaacaaaggttcagaaaacgaaaaaaaaaag
6	atcctattttacgaaaacaaataagggttcagaagaaagcgagaataaaaaaag
7	atcctgttttcagaaaaaagggttcagaaagcgagactcaaaaatg
8	atcctattttccacaaacacaag
9	atcctgttttcagaaaacaaaaaagggttcagaaagcgagactcaaaaatg
10	atcctgttttacgagaacaaacaagacaagggcttacaaaaaaggggagg
11	atcctgttttccgaaaacaaacaaaggttcagaaagcgaaaataaaaaaag
12	atcctgttttcagaaaacaagggttcagaaagcgagaaccaaaaaaag
13	atcctattttatgaaaaccaacaaaaaacaaaacaacaaagattcaaagaataaaaaag
14	atcctgttttccgaaaacaaacaaaggttcataaagacaga
15	atcctgttttacgaaaaccaaaaagagttcagaaagagaaagggagcataaaaaaag

16	atcctgttttctgaaaacaaacaaaacaagggttcataaagatagaataaaaaag
17	atcctattttacgagaacaaaaacaaacaaggggtcagaacgggagaaaaaaag
18	atcctattttcaaaaaaaaagttcagaaagatcgaataaaaaaag
19	atcctgttttcccaaaacaaaggttcagaaagaaaaaag
20	atcctgttttacgagaacaaacaagggttcagaacagaacgcgagaaaaag
21	atcctgttttacaagaacaaataagggttcagaaagcgaaaaaggg
22	atcctattttccacaaacaaaggttcagaaaacgaaaacaag
23	atcctgttttccgaaaaccaaaaagagttcaaagagttagaataaaaag
24	atcccgttttatgaaaacaaaacaaacaaggggttcagaaagtgagaaaagg
25	atcctattgtccgaaaacaaagaaagattcagaaagcaagaataacacaag
26	atcctgtgttcagcaaacaaggttcagaaagcgagactcaaaaatg
27	atcccgttttccgaaaccaaaggttcagaaagtgaaaaaag
28	atcctgattttcgaaaacaaacaaagattcagaaagcgaaaataaaaaaag
29	atcctgttttccgaaaaccaagaagagttcagaaaggggagaatcaaataaaaaagg
30*	atcctgttttcgaaaataaacaaagattcagaaagcgaaaataaaaaaag
31	atcctgttttccaaaaacaaacaaaggttcgtatcataaagatagaataaaaaag
32	atccggttttctgaaaacaaacaagggttcagaaagcgataataaaaaag
33	atcctattttacgaaaacaaataagggttcagaaagcgagactcaaaaatg
34	atcctgttttccgaaaccaaaggttcagaaagttaaaaagg
35	atcctgttttccgaaaacaaacaaaagttaagaaaaag
36	atcctgttttccgaaaacaaacaaaggttcagaaagcgaaaatcaaaaaag
37	atcctgttttccgaaaacaaaggttcagaagcgaaaaagg
38	atcctgttttctgaaaacaaacaaaggttcagaaagcgataataaaataaaaaaggatagatagg
39	atccggttttctgaaaacaaacaagggttcagaaggcgataataaaaaaggatag
40	atcctgttttccgaaaccaaaggttcagaaagtgaaaaagg
41	atcctgttttccgaaaacaaccaagggttcagaaaacgataataaaaaaag
42	atcctattttacgaaaaccaacaaaacaacaaagattcagaataaaaaaag
43	atcctgttttccgaaaacaaacaagagttcataaagacagaataaaaaaaag
44	atccgtttttttgaaaacaaagttcataaagacagaataaaaaag
45	atcccgttttccgaaaacaaagaaaagttcagaaagcgataaaaaaaagg
46	atcctattttcaaaaaaaaaagttcagaaagctcgaataaaaaaag
47	atcctgctttccgaaaacaaagaaaagttcagaaagcgagaatcaaaaaag
48	atcttgttttccgaaaccaaacaaagattcataaagaaagacagaacaaaaag
49	atcccgttttccgaaaccaaaggttcagaaagtgaaaaggg
50	atcctgctttacgcgaacaaaaaagggtttagaaagcgagaaaaaaaaagggtttagaaagcgagaaaaaaaagg
51	atcctgtttaaaaaagcgttcaaaaagaaatag
52	atcctgttttccgaaaacaaacaaaggttcagaaggcgaaaaaagg
53	atcctattttatgaaaaccaacaaaacaacaaagattcagaataaaaaaag
54	atcctattttatgaaaacggacaaaacaacaaagattcataaagcaagaataaaaaaag
55	atcctgttttccgaaaaccaagaagagttcagaaagggaaaatcaaataaaaaagg
56	atcctattattttacgaaaataaaaaaaggttcagcaagcgagaataataaaaaaag
57	atcctgttttccgaaaacaaacaaaggttcagcaagcgagaataataaaaaaag
58	atcctgttttctgaaaacaaggattcagaaagtgataataaaaaag
59	atcctgtttttgttttccgaaaaaaaaaaaacaaagattgataaagaaaaaacataaaacataaaaaaagaaagaaaaaag
60	atccggttttctgaaaacaaacaaggattcaggattcagaaagcgataataaaaaagaatcg
61	atcctgttttccgaaaaccaagaagagtttagaaagggagaataaaaaaag
62	atcctgttttacgaaaacaaataagggttcataaatacagaataaaaaaag
63	atcctgtttttcgaaaacaaacaaagattcataaagcgaaaaaaaaag
64	atcctgttttacgagaacaaacaagggttcagaaagcgaaaaaggg
65	atcctgttttccgaaaacaagagcaagggtttataaagtgagaaaag
66	atcctgttttcagaaaacaagggttcagaaagcgagaaccaaaaaaaaaaag
67	atcctgttttcagaaaacaagggttcagaaagcgagaaccaaaaaaaaaag
68	atcctgttttacgagaacaaacaagggttcagaacacgagaaaggg
69	atcctgctttacacgaacaaaaaagggtttagaaagcgagaaaaaaaaaggg
70	atcctgttttccgaaaacaaacaaaggtttagaaagcaagaataaaaaag
71	atcctatttttcgaaaaaaaacaaaagttcagaaaaaag
72	atcctgttttccgaaaacaaacaaaggttcataaagacagaaataaagg
73	atcctgttttccgaaaacaaaggttcgtaaagatagaataaaaaag

74	atcctgttttccgaaaccaaagaaaacaaagaagagttcagaaagcaagaataaaaaaag
75	atcctgttttcaaaaaacaaacaaaggttcataaagacagaaataaagg
76	atcctgttttacgaaaacaaacaaaaaaaggtttctaaagacagaataaaaaag
77	atccttattttgagaaaacaaaggtttataaaactagaatttaaaag
78	atcctgttttccccaaacaagagttcagaaagaaaaaagg
79	atcctgttttcaaaaaacaaacaaaggttcataaagacaga
80	atcctgttttccaaaaccaaaggttcagaaagtgaaaatgaaaaagg
81	atcccgttttatgaaaacaaaacaagcaggggttcagaaagtgagaaaagg
82	atctttattttgataaaatgggggtttaatttataaaactagaatcaaaaagg
83	atcctgtgttcagaaaacaaggttcagaaggcgagaatcaaaaaag
84	atcctgttttctgaaaacaaacaaaggttcagaaacagaaagcgagaatcaaaaaag
85	atcctgttttccgaaaaccaaaaagagttcaaaaagtgagaataaaaag
86	atcctgttgttttctcaaaacaaagattcaaaaaacgaaaaataaag
87	atcctattattttacgaaaataaaaaaaggttcagaaagcgaaaatcaaaaaag
88	atccttttttacaaaaacaaacaagggttcagaaagcgaaaaaaaaaaagg
89	atcctgttttccgaaaccaaacaaaggtcagaaagcgaaaaaaaaaag
90	atctttattttgagaaaacaagggtttataaaactagaataaaaaaag
91	atcctattttacgaaaacaaaaaagggttcagaaagcgagactcaaaaatg
92	atcctgtttttgttttccgaaaaaaaaaaaacaaagattgataaagaaaaaacataaaaaaagaaagaaaaaaaaag
93	atccttttttctcaaaacaaaggttcagaaagcgagactcaaaaatg
94	atcctgtttttcgaaaacaaacaaaggttcataaagacagaagaaaaaaagaaag
95	atcctattttccaaaaaaaagttcagaaagctcgaataaaaaaag
96	atcctgctttccgaaaacaaagaaaagttcagaaagatcgaataaaaaaag
97	atcctgttttattaaaacaaacaagggtttcataaaccgagaataaaaaag
98	atcctattttcaggttcagaaaacgaaaacaag
99	atccttttttctcaaaacaaaggttcagaaaacgaaaaaaaaag
100	atcctgttttacgagaacaaacaagcgttcagaacacgagaaaggg
101	atcctgttttccgaaaacaaacaaagacaaaggttcataaagacagaattcaaaaaaaagg
102	atcctgttttccgaaaacaaagaataaagaagagttcagaaagcgagaataaaaaatcaaag
103	atcctgttgttttctcaaaacaaagattcaaaaaacgaaaaataaaaag
104	atcctgttttctcaaaacaaaggttcaaaagacgaaaaaaag
105	atcctgttttccccaaacaagagttcagaaagaaaaaggg
106	atcctgttttcccaaaacaaagtttcagaaagaaaaaaag
107	atcctattttccacaaacaaaggttcagaaaatgaaaacaag
108	atcccgttttatgaaaacaaaacaaacaggggttcagaaagcgaaaaaggg
109	atcctgttttacaagaacaaataagggttcagaacagaacgcgagaaaaag
110	atcctggtttccgaaaaaagttcatacattacataaagacagaatcaaaaag

*Sequence no. 30 is the same sequence as the one identified in the positive control for *Manilkara zapota* (Sapotaceae).

Appendix D. One-Way Analysis of Variance (ANOVA) for the mean number of sequences detected in BSD samples collected from different sites.

ANOVA

Sites

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	28.371	3	9.457	.666	.579
Within Groups	454.379	32	14.199		
Total	482.750	35			

Appendix E. Unique sequences (corresponding to Tables 5.6a and b) identified in BSD samples from Ta Kou Nature Reserve.

Sequence no.	Sequences
1	atcctgtgttcagcaaacaaggttcagaaagcgagaatcaaaaaaag
2	atccttttttctcaaaacaaaggttcagaaaacgaaaaaaaaaag
3^	atcctattttacgaaaacaaataagggttcagaagaaagcgagaataaaaaaag
4	atccggttttctgaaaacaaacaagggttcagaaggcgataataaaaaag
5	atcctgttttctcaaaacaaaacaaaggttcaaaaaacaaaaaaaag
6	atcctgttttcagaaaaaagggttcagaaagcgagactcaaaaatg
7^	atcctgttttacgagaacaaacaagacaagggcttacaaaaaaggggagg
8^	atcctgttttcagaaaacaaaaaagggttcagaaagcgagactcaaaaatg
9	atcctattttccacaaacacaag
10^	atcctgttttctgaaaacaaacaaaacaagggttcataaagatagaataaaaaag
11	atcctattttatgaaaaccaacaaaaaacaaaacaacaaagattcaaagaataaaaaag
12^	atcctattttacgagaacaaaaacaaacaaggggtcagaacgggagaaaaaaag
13^	atcctgttttccgaaaacaaacaaaggttcagaaagcgaaaataaaaaaag
14	atcctgtgttcagaaaacaaggttcagaaagcgagaatcaaaaacagaaaaag
15	atcctgttttacgaaaaccaaaaagagttcagaaagagaaagggagcataaaaaaag
16	atcctgttttcagaaaacaagggttcagaaagcgagaaccaaaaaaag
17^	atcctgtgttcagcaaacaaggttcagaaagcgagactcaaaaatg
18^	atcctattttacgaaaacaaataagggttcagaaagcgagactcaaaaatg
19^	atcctattttcaaaaaaaaaagttcagaaagctcgaataaaaaaag
20^	atcccgttttccgaaaacaaagaaaagttcagaaagcgataaaaaaagg
21	atcctattttcaaaaaaaaagttcagaaagatcgaataaaaaaag
22^	atcctgttttccgaaaacaaacaaaggttcagcaagcgagaataataaaaaaag
23^	atcctattattttacgaaaataaaaaaaggttcagcaagcgagaataataaaaaaag
24^	atcctgttttctgaaaacaaggattcagaaagtgataataaaaaag
25^	atcctgtttttgttttccgaaaaaaaaaaaacaaagattgataaagaaaaaacataaaacataaaaaaagaaagaaaaaaag
26	atcctgttttccgaaaacaaacaaaggttcagaaagcgaaaatcaaaaaag
27^	atcctgtgttcagaaaacaaggttcagaaggcgagaatcaaaaaag
28	atcctattttccacaaacaaaggttcagaaaacgaaaacaag
29	atcctgttttccgaaaacaaacaaaggttcagaaggcgaaaaaagg
30^	atctttattttgagaaaacaagggtttataaaactagaataaaaaaag
31	atccggttttctgaaaacaaacaagggttcagaaggcgataataaaaaaggatag
32	atcctattttatgaaaaccaacaaaacaacaaagattcagaataaaaaaag
33*	atcctgttttcgaaaataaacaaagattcagaaagcgaaaataaaaaaag
34^	atcctgttttctgaaaacaaacaaaggttcagaaacagaaagcgagaatcaaaaaag
35^	atcctgttttccgaaaaccaaaaagagttcaaaaagtgagaataaaaag
36	atccggttttctgaaaacaaacaagggttcagaaagcgataataaaaaag
37^	atcctattttacgaaaacaaaaaagggttcagaaagcgagactcaaaaatg
38^	atcctgttttccgaaaccaaacaaaggtcagaaagcgaaaaaaaaaag
39	atcctattgtccgaaaacaaagaaagattcagaaagcaagaataacacaag
40	atcctattttatgaaaacggacaaaacaacaaagattcataaagcaagaataaaaaaag
41^	atcctattattttacgaaaataaaaaaaggttcagaaagcgaaaatcaaaaaag
42^	atcctttttttctcaaaacaaaggttcagaaagcgagactcaaaaatg
43^	atcctgtttttgttttccgaaaaaaaaaaaacaaagattgataaagaaaaaacataaaaaaagaaagaaaaaaaag
44^	atcctgttgttttctcaaaacaaagattcaaaaaacgaaaaataaag
45^	atcctttttttacaaaaacaaacaagggttcagaaagcgaaaaaaaaaaagg

*Sequence no. 33 is the same sequence as the one identified in the positive control for *Manilkara zapota* (Sapotaceae).

^sequences only found in Ta Kou Nature Reserve.

Appendix F. Unique sequences (corresponding to Tables 5.7a and b) identified in BSD samples from Cat Tien National Park.

Sequence no.	Sequences
1^	atcctgttttacgagaacaaacaagggttcagaacagaacgcgagaaaaag
2^	atcctgttttacaagaacaaataagggttcagaaagcgaaaaaggg
3	atcctgttttcccaaaacaaaggttcagaaagaaaaaag
4	atcctgtgttcagaaaacaaggttcagaaagcgagaatcaaaaacagaaaaag
5	atcctgttttccgaaaacaaacaaaggttcataaagacaga
6	atcctattgtccgaaaacaaagaaagattcagaaagcaagaataacacaag
7	atcccgttttatgaaaacaaaacaaacaggggttcagaaagtgagaaaagg
8	atccggttttctgaaaacaaacaagggttcagaaggcgataataaaaaag
9	atcctattttccacaaacaaaggttcagaaaacgaaaacaag
10	atcctgtgttcagcaaacaaggttcagaaagcgagaatcaaaaaaag
11	atcctgttttccgaaaaccaagaagagttcagaaagggagaatcaaataaaaaagg
12	atcctgttttacgaaaaccaaaaagagttcagaaagagaaagggagcataaaaaaag
13	atccggttttctgaaaacaaacaagggttcagaaagcgataataaaaaag
14^	atcctgctttacgcgaacaaaaaagggtttagaaagcgagaaaaaaaagggtttagaaagcgagaaaaaaaagg
15^	atcctgtttaaaaaagcgttcaaaaagaaatag
16	atcctgttttccgaaaacaaacaaaagttaagaaaaag
17	atcctgttttccaaaaacaaacaaaggttcgtatcataaagatagaataaaaaag
18^	atcctgttttccgaaaacaagagcaagggtttataaagtgagaaaag
19^	atcctgttttacgagaacaaacaagggttcagaaagcgaaaaaggg
20^	atcctgttttacgaaaacaaataagggttcataaatacagaataaaaaaaag
21^	atcctgtttttcgaaaacaaacaaagattcataaagcgaaaaaaaaaag
22	atcctgttttcagaaaacaagggttcagaaagcgagaaccaaaaaaag
23*	atcctgttttcgaaaataaacaaagattcagaaagcgaaaataaaaaaag
24	atccttttttctcaaaacaaaggttcagaaaacgaaaaaaaaaag
25	atcctgttttcagaaaaaagggttcagaaagcgagactcaaaaatg
26	atcctattttatgaaaaccaacaaaaaacaaaacaacaaagattcaaagaataaaaaag
27	atcctattttatgaaaacggacaaaacaacaaagattcataaagcaagaataaaaaaag
28	atcctgttttccgaaaacaaacaaaggttcagaaggcgaaaaaagg
29	atcctgattttcgaaaacaaacaaagattcagaaagcgaaaataaaaaaag
30	atcctgttttccgaaaccaaaggttcagaaagtgaaaaagg
31	atcctgttttccgaaaacaaaggttcagaagcgaaaaagg
32^	atcctgttttcccaaaacaaagtttcagaaagaaaaaaag
33^	atcctgttttccgaaaacaaagaataaagaagagttcagaaagcgagaataaaaaatcaaag
34^	atcctgttttacaagaacaaataagggttcagaacagaacgcgagaaaaag
35^	atcctattttccacaaacaaaggttcagaaaatgaaaacaag
36^	atcctgttttccccaaacaagagttcagaaagaaaaaggg
37^	atcctgttgttttctcaaaacaaagattcaaaaaacgaaaaataaaaag
38^	atcccgttttatgaaaacaaaacaaacaaggggttcagaaagcgaaaaaggg
39^	atcctggtttccgaaaaaagttcatacattacataaagacagaatcaaaaag
40^	atccttttttctcaaaacaaaggttcagaaaacgaaaaaaaaag
41^	atcctgttttacgagaacaaacaagcgttcagaacacgagaaaggg
42^	atcctgttttctcaaaacaaaggttcaaaagacgaaaaaaag
43^	atcctattttcaggttcagaaaacgaaaacaag
44^	atcctgttttccgaaaacaaacaaagacaaaggttcataaagacagaattcaaaaaaaagg

*Sequence no. 23 is the same sequence as the one identified in the positive control for *Manilkara zapota* (Sapotaceae).

^sequences only found in Cat Tien National Park.

Appendix G. Unique sequences (corresponding to Table 5.8) identified in BSD samples from Nui Chua National Park.

Sequence no.	Sequences
1	atcctgttttccgaaaacaaacaaaggttcataaagacaga
2	atccggttttctgaaaacaaacaagggttcagaaggcgataataaaaaag
3	atcctgtgttcagaaaacaaggttcagaaagcgagaatcaaaaacagaaaaag
4	atcctgattttcgaaaacaaacaaagattcagaaagcgaaaataaaaaaag
5^	atccgtttttttgaaaacaaagttcataaagacagaataaaaaag
6^	atcctattttacgaaaaccaacaaaacaacaaagattcagaataaaaaaag
7^	atcctgttttccgaaaacaaacaagagttcataaagacagaataaaaaaaag
8	atcctgttttctcaaaacaaaacaaaggttcaaaaaacaaaaaaaag
9	atcctgttttccgaaaaccaaaaagagttcaaagagttagaataaaaag
10	atcctgttttctgaaaacaaacaaaggttcagaaagcgataataaaataaaaaaggatagatagg
11	atcctgttttccaaaaacaaacaaaggttcgtatcataaagatagaataaaaaag
12	atcctgttttccgaaaccaaaggttcagaaagtgaaaaagg
13	atcctgttttccgaaaacaaaggttcagaagcgaaaaagg
14	atcctgttttcagaaaacaagggttcagaaagcgagaaccaaaaaaag
15	atcctattttccacaaacaaaggttcagaaaacgaaaacaag
16*	atcctgttttcgaaaataaacaaagattcagaaagcgaaaataaaaaaag
17	atcctattttcaaaaaaaaagttcagaaagatcgaataaaaaaag
18	atcctgtgttcagcaaacaaggttcagaaagcgagaatcaaaaaaag
19	atccttttttctcaaaacaaaggttcagaaaacgaaaaaaaaaag
20	atcccgttttccgaaaccaaaggttcagaaagtgaaaaaag
21	atcctgttttccgaaaacaaccaagggttcagaaaacgataataaaaaaag
22	atcctgttttccgaaaaccaagaagagttcagaaagggaaaatcaaataaaaaagg
23	atcctgttttccgaaaacaaacaaaggttcagaaagcgaaaatcaaaaaag
24	atcctattttatgaaaaccaacaaaacaacaaagattcagaataaaaaaag
25	atcccgttttatgaaaacaaaacaaacaggggttcagaaagtgagaaaagg
26^	atcctgttttacgaaaacaaacaaaaaaaggtttctaaagacagaataaaaaag
27^	atcccgttttatgaaaacaaaacaagcaggggttcagaaagtgagaaaagg
28^	atcctgttttcagaaaacaagggttcagaaagcgagaaccaaaaaaaaaaag
29^	atcctgttttcagaaaacaagggttcagaaagcgagaaccaaaaaaaaaag
30^	atcctgttttacgagaacaaacaagggttcagaacacgagaaaggg
31^	atcctgttttccgaaaacaaacaaaggtttagaaagcaagaataaaaaag
32^	atcctgttttccaaaaccaaaggttcagaaagtgaaaatgaaaaagg
33^	atcctgttttccgaaaacaaacaaaggttcataaagacagaaataaagg
34^	atcctgttttccgaaaacaaaggttcgtaaagatagaataaaaaag
35^	atcctgttttcaaaaaacaaacaaaggttcataaagacaga
36^	atcctgttttcaaaaaacaaacaaaggttcataaagacagaaataaagg
37^	atcctgctttacacgaacaaaaaagggtttagaaagcgagaaaaaaaaaggg
38^	atctttattttgataaaatgggggtttaatttataaaactagaatcaaaaagg
39^	atcctattttttcgaaaaaaaacaaaagttcagaaaaaag
40^	atcctgttttccccaaacaagagttcagaaagaaaaaagg
41^	atcctgttttccgaaaccaaagaaaacaaagaagagttcagaaagcaagaataaaaaaag
42^	atccttattttgagaaaacaaaggtttataaaactagaatttaaaag

*Sequence no. 16 is the same sequence as the one identified in the positive control for *Manilkara zapota* (Sapotaceae).

^sequences only found in Nui Chua National Park.

Appendix H. Unique sequences (corresponding to Table 5.9) identified in BSD samples from Hon Heo Mountain.

Sequence no.	Sequences
1	atccggttttctgaaaacaaacaagggttcagaaggcgataataaaaaag
2	atcctgtgttcagaaaacaaggttcagaaagcgagaatcaaaaacagaaaaag
3	atcctattttcaaaaaaaaagttcagaaagatcgaataaaaaaag
4	atcctattttccacaaacacaag
5	atcctgttttccgaaaaccaaaaagagttcaaagagttagaataaaaag
6	atcccgttttccgaaaccaaaggttcagaaagtgaaaaaag
7^	atcctgttttccgaaaccaaaggttcagaaagttaaaaagg
8	atcctgttttctcaaaacaaaacaaaggttcaaaaaacaaaaaaaag
9	atcctgttttcagaaaacaagggttcagaaagcgagaaccaaaaaaag
10^	atcttgttttccgaaaccaaacaaagattcataaagaaagacagaacaaaaag
11^	atcccgttttccgaaaccaaaggttcagaaagtgaaaaggg
12^	atcctgctttccgaaaacaaagaaaagttcagaaagcgagaatcaaaaaag
13	atccggttttctgaaaacaaacaagggttcagaaggcgataataaaaaaggatag
14	atcctgttttccgaaaaccaagaagagttcagaaagggagaatcaaataaaaaagg
15	atcctgttttccgaaaacaaccaagggttcagaaaacgataataaaaaaag
16^	atcctgttttccgaaaaccaagaagagtttagaaagggagaataaaaaaag
17^	atccggttttctgaaaacaaacaaggattcaggattcagaaagcgataataaaaaagaatcg
18	atcctattttccacaaacaaaggttcagaaaacgaaaacaag
19*	atcctgttttcgaaaataaacaaagattcagaaagcgaaaataaaaaaag
20	atcctgttttccgaaaacaaacaaaggttcataaagacaga
21	atcctgttttcccaaaacaaaggttcagaaagaaaaaag
22	atcctgttttccgaaaacaaacaaaagttaagaaaaag
23	atcctgttttctgaaaacaaacaaaggttcagaaagcgataataaaataaaaaaggatagatagg
24	atcctgttttccgaaaaccaagaagagttcagaaagggaaaatcaaataaaaaagg
25^	atcctgtttttcgaaaacaaacaaaggttcataaagacagaagaaaaaaagaaag
26^	atcctgttttattaaaacaaacaagggtttcataaaccgagaataaaaaag
27^	atcctattttccaaaaaaaagttcagaaagctcgaataaaaaaag
28^	atcctgctttccgaaaacaaagaaaagttcagaaagatcgaataaaaaaag

*Sequence no. 19 is the same sequence as the one identified in the positive control for *Manilkara zapota* (Sapotaceae).
^sequences only found in Hon Heo Mountain.

Appendix I. Food plant taxa of wild black-shanked doucs in Vietnam and Cambodia based on field observations from published studies (Hoang Minh Duc 2007; Rawson 2009; O'Brien 2014) and diet metabarcoding from current research (only taxa identified to at least species level are listed here).

No.	Family	Species
1	Anacardiaceae	*Bouea oppositifolia*
2		*Buchanaria* cf. *siamensis*
3		*Lannea coromandelica*
4		*Pentaspadon* aff. *poilanei*
5		*Semecarpus perniciosa*
6		*Spondias pinnata*
7		*Swintonia* aff. *minuta*
8		*Swintonia floribunda*
9	Annonaceae	*Alphonsea gaudichaudiana*
10		*Cyathocalyx annamensis*
11		*Polyalthia* cf. *cerasoides*
12		*Uvaria fauveliana*
13	Apocynaceae	*Alstonia angustifolia*
14		*Alstonia macrophylla*
15		*Holarrhena pubescens*
16		*Melodinus myrtifolius*

17		*Parameria laevigata*
18	Aquifoliaceae	*Ilex umbellata*
19	Araliaceae	*Schefflera canaensis*
20		*Schefflera octophylla*
21	Avagaceae	*Dracaena cochinchinensis*
22	Burseraceae	*Bursera* cf. *serrata*
23		*Canarium subulatum*
24	Cannabaceae	*Celtis biondii*
25	Combretaceae	*Anogeissus acuminata*
26		*Terminalia franchetii*
27	Dilleniaceae	*Dillenia blanchardii*
28	Dipterocarpaceae	*Dipterocarpus alatus*
29		*Hopea ferrea*
30		*Hopea odorata*
31	Euphorbiaceae	*Bischofia javanica*
32	Fabaceae	*Acacia concinna*
33		*Acacia tenuifolia*
34		*Afzelia xylocarpa*
35		*Albizia myriophylla*
36		*Bauhinia championii championii*
37		*Bauhinia corymbosa*
38		*Dialium cochinchinensis*
39		*Entada pursaetha*
40		*Peltophorum* cf. *dasyrrhachis*
41		*Peltophorum pterocarpum*
42		*Sindora siamensis*
43		*Xylia xylocarpa*
44	Gnetaceae	*Gnetum latifolium* var. *funicculare*
45	Guttiferae	*Callophyllum ceriferum*
46		*Garcinia aff. harmandii*
47		*Garcinia cf. merguensis*
48		*Garcinia oligantha*
49		*Garcinia schefferi*
50	Irvingiaceae	*Irvingia malayana*
51	Lamiaceae	*Vitex canescens*
52		*Vitex* cf. *leptobotrys*
53		*Vitex peduncularis*
54		*Vitex pinnata* var. *ptilota*
55	Lecythidaceae	*Barringtonia acutangula*
56		*Careya arborea*
57	Loganiaceae	*Strychnos minor*
58	Lythraceae	*Lagerstroemia* cf. *calyculata*
59	Malvaceae	*Firmiana simplex*
60	Meliaceae	*Aglaia euphoroides*
61		*Amoora gigantean*
62		*Heynea velutina*
63	Mimosoideae	*Albizia corniculata*
64		*Archidendron balansae*
65		*Archidendron chevalieri*
66		*Archidendron pellitum*
67	Moraceae	*Artocarpus rigida asperulus*
68		*Ficus altissima*
69		*Ficus callophylla var. callophylla*
70		*Ficus curtipes*

71		*Ficus depressa*
72		*Ficus cf. lamponga*
73		*Ficus phanrangensis*
74		*Ficus racemose*
75		*Ficus* cf. *subgelderi*
76		*Ficus superba* var. *japonica*
77		*Ficus tinctoria*
78		*Ficus tjakela*
79	Myrtaceae	*Cleistocalyx nigrans*
80		*Syzygium formosum* var. *ternifolium*
81		*Syzygium* cf. *grandis*
82		*Syzygium levinei*
83		*Syzygium pachysarcum*
84		*Syzygium ripicola*
85	Olacaceae	*Olax acuminata*
86	Pandanaceae	*Pandanus cornifer*
87	Papilionoideae	*Millettia reticulata*
88		*Ormosia* cf. *poilanei*
89	Poaceae	*Bambusa procea*
90	Podocarpaceae	*Podocarpus neriifolius*
91	Rhamnaceae	*Ziziphus cambodiana*
92	Rhizophoraceae	*Carallia lancaeifolia*
93	Rubiaceae	*Haldina cordifolia*
94		*Morinda tomentosa*
95		*Tarenna asiatica*
96	Rutaceae	*Zanthoxylum rhetsa*
97	Sapindaceae	*Nephelium hypoleucum*
98		*Nephelium melliferum*
99		*Xerospermum noronhianum*
100	Sapotaceae	*Pouteria* cf. *obovata*
101	Sterculiaceae	*Sterculia lissophylla*
102	Symplocaceae	*Symplocos adenophylla* var. *touranensis*
103		*Symplocos* aff. *guillauminii*
104	Tetramelaceae	*Tetrameles nudiflora*
105	Theaceae	*Gordonia axillaris*

Appendix J. One-Way ANOVA for the mean number of sequences detected in ISL samples collected from different sites.

ANOVA

Sites

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	40.879	3	13.626	1.797	.165
Within Groups	273.021	36	7.584		
Total	313.900	39			

Appendix K. Unique sequences (corresponding to Tables 6.4a and b) identified in ISL samples.

Sequence no.	**Sequences**
1	atccggttttctgaaaacaaacaagggttcagaaggcgataataaaaaag
2	atcctattttatgaaaaccaacaaaaaacaaaacaacaaagattcaaagaataaaaaag
3	atcctgtgttcagaaaacaaggttcagaaagcgagaatcaaaaacagaaaaag
4	atcctgttttccgaaaaccagaaaacgctaataaaaagg

5	atcctattttccacaaacaaaggttcagaaaacgaaaacaag
6	atccttgttttgagaaaaag
7^	atcctgttttccgaaaaccaaaaagagttcaaaaagtgagaataaaaag
8	atccggttttccaaaaacaaacaaaggttcgtatcataaagatagaataaataaag
9	atcctgttttccgaaaacaaacaaaggttcataaagacaga
10	atcctgttttccgaaaagcaagaagagttcagaaagggagaatcaaaataaaaaaag
11	atcctattttccaaaaaaaaagttcagaaagctcgaataaaaaaag
12	atcctgttttctcaaaacaaaggttcaaaagacgaaaaaaag
13	atcctgattttcgaaaacaaacaaagattcagaaagcgaaaataaaaaaag
14	atccggttttctgaaaacaaacaagggttcagaaagcgataataaaaaag
15^	atcctgttttacgaaaacaaacaaaaaaaggtttctaaagacagaataaaaaag
16	atcctgttttacgagaacaaacaagcgttcagaacacgagaaaggg
17	atcccaaaacaaacagaggggttcagaaagtgaaaaaggg
18	atcctgttttctgaaaacaaacaaaggttcagaaagcgataataaaataaaaaaggatagatagg
19	atccgtttttttgaaaacaaagttcataaagacagaataaaaaag
20	atccagtttacagagacaatagtttccttgactaggaagagaaag
21#	atcctgttttccaaaaacaaacaaaggttcgtatcataaagatagaataaaaaag
22	atcctgtttttcgaaaaccaaaaagagttcaaaaagtgagaataaaag
23#	atcctatttattattttacgaaaataaacattttacgaaaataaacaaaggttcagcaagcgagaataataaaaaaag
24#	atcctattttacacaaacaaaggttcagaaaacgaaaacaag
25	atcctgttttacgagaacaaacaagggttcagaacgcgagaaaaag
26^	atcctgtttttagaaaaaaaggggttcagaaagcgagactcag
27^	atcctgttttccgaaaccaaaggtgaaaaggg
28*	atcctgttttcgaaaataaacaaagattcagaaagcgaaaataaaaaaag
29#	atcctgtgttcagaaaacaaggttcagaaagcgagaatcaaaaaaag
30^	atcctattttatgaaaaccaacaaaacaacaaagattcagaataaaaaaag
31^	atcctgttttccgaaaacaaacaaagttcaaaaagacagaataaaaaaag
32	atcctgttttccccaaacaagagttcagaaagaaaaaagg
33^	atccggttttctgaaaacaaacaagggttcagaaggcgataataaaaaaggatag
34#	atcccgttttccgaaaccaaaggttcagaaagtgaaaaggg
35	atcctgttttccgaaaccaaaggttcagaaagtgaaaaggg
36	atcctgttttccgaaaagcaagaagagttcagaaaggggagaatcaaaaacagaaaaag
37^	atcctattttccaaaaaaaaaagttcagaaagctcgaataaaaaaag
38^	atccggttttctgaaaacaaacaagggttcagaaagctcgaataaaaaaag
39^	atcctgttttccgaaaatgagtagtgaaagcaagctagaataaaaaaag
40^	atcctattttcaggttgagaaaacgaaaacgaaaacaag
41^	atcctattttcaggttcagaaaacgaaaacaag
42+	atcctgttttccgaaaacaaacaaaagttcagaaaaaaag
43!	atccttttttccgaaaacaaaaaaaaag
44#	atcctgttttccgaaaagcaagaagagttcagaaaggggagaatcaaaaaaag
45#	atcctgttttccgaaaagcaagaagagttcagaaggcgataataaaaaag
46#	atcctgttttccgaaaagcaagaagagttcagaaagcgagaatcaaaaacagaaaaag
47#	atcctgttgttttctcaaaacaaagattcaaaaaacgaaaaataaaaag
48^	atcctgttttcagaaaacaagggttcagaaagcgagaaccaaaaaaag
49^	atcctattatttttattttacgaaaataaacaagggttcagcaagcgagaataataaaaaaag
50^	atcctattattttacgaaaagaaacattttacaaaaatcaacaaaggttcagcaagcgagaataataaaaaaag
51+	atcctgttttcagaaaacaaacaaaaaaggggttcagaaaaggagactcaaaagtg
52!	atccggttttctgaaaacaaacaagggttcagaaagcgagaatcaaaaacagaaaaag
53^	atccggttttctgaaaacaaacaagggttcagaaagcgaaaataaaaaaag
54!	atcctgttttctgaaaacaaacaaaggttcagaaagcgataataaaaaag
55^	atcctgattttcgaaaacaaacaaagattcagaaagcgataataaaaaag
56^	atccggttttctgaaaacaaacaaagattcagaaagcgaaaataaaaaaag
57#	atcctattttccacaaacaaaggttcaaaagacgaaaaaaag
58^	atcctgattttcgaaaacaaacaaagattcagaaagctcgaataaaaaaag
59^	atccggttttccaaaaacaaacaagggttcagaaggcgataataaaaaag
60^	atcctgattttcgaaaacaaacaagggttcagaaagcgataataaaaaag
61^	atcctgattttcgaaaacaaacaagggttcagaaggcgataataaaaaag
62#	atcccgttttatgaaaacaaaacaaacaaggggttcagaaagtgagaaaagg

63^	atcctcttttccaagagcaaacaggggttcagaaagcgaaaaaggg
64[#]	atcctgttttccgaaaaccagaaaacgctaataaaaaaag
65[#]	atctttatttgctagaatcaaaaatcaaaaag
66^	atcctgttttacgaaaaccacccgcggtttataaagcgcgaatacaaaaagaatag
67[!]	atccggttttctgaaaacaaacaagggttcagaaggcgataataaaataaaaaaggatagatagg
68^	atcccgttttatgaaaacaaaacaaacaggggttcagaaagttataattataaaagg

*Sequence no. 28 is the same sequence as the one identified in the positive control for *Manilkara zapota* (Sapotaceae); ^sequences only found in Chua Hang hill; [#]sequences only found in Khoe La hill; [!]sequences only found in Lo Coc hill; [+]sequences only found in Mo So hill

Appendix L. Food plant taxa of wild Indochinese silvered langurs in Vietnam based on field observations from published studies (Hoang Minh Duc *et al.* 2010c; Le Hong Thia *et al.* 2015) and diet metabarcoding from current research (only taxa identified to at least species level are listed here).

No.	**Family**	**Species**
1	Acanthaceae	*Avicennia alba*
2		*Avicennia officinalis*
3	Agavaceae	*Dracaena cambodiana*
4	Anacardiaceae	*Lannea coromandelica*
5		*Mangifera indica*
6		*Spondias pimata*
7	Annonaceae	*Sageraea elliptica*
8	Apocynaceae	*Gymnanthera oblonga*
9		*Sarcostemma acidum*
10	Araceae	*Rhaphidophora hongkongensis*
11		*Scindapsus* cf. *officinalis*
12	Arecaceae	*Caryota mitis*
13	Capparaceae	*Capparis micrantha micrantha*
14	Combretaceae	*Anogeisssus acuminata*
15		*Combretum tetralophum*
16		*Terminalia catappa*
17		*Terminalia triptera*
18	Commelinaceae	*Commelina salicifolia*
19	Cucurbitaceae	*Coccinia grandis*
20	Cycadaceae	*Cycas clivicola*
21	Dioscoreaceae	*Dioscorea bulbifera*
22		*Dioscorea esculenta*
23	Ebenaceae	*Diospyros mollis*
24	Euphorbiaceae	*Bridelia monoica*
25		*Glochidion littorale*
26		*Phyllathus reticulatus*
27		*Sauropus villosus*
28	Fabaceae	*Bauhinia bracteata bracteata*
29		*Bauhinia purpurea*
30		*Derris trifolia*
31		*Leucoena leucocephala*
32		*Pithecellobium dulce*
33		*Tamarindus indica*
34	Flagellariaceae	*Flagellaria indica*
35	Gnetaceae	*Gnetum gnemon*
36	Lamiaceae	*Vitex negundo*
37	Lauraceae	*Persea americana*
38	Loranthaceae	*Dendrophthoe pentandra*
39		*Taxillus chinensis*

40	Malvaceae	*Ceiba pentandra*
41		*Grewia tomentosa*
42		*Sterculia stigmarota*
43	Melastomaceae	*Memecylon caeruleum*
44	Menispermaceae	*Stephania dielsiana*
45		*Tinospora cordifolia*
46		*Tinospora crispa*
47	Moraceae	*Ficus aurantiaca*
48		*Ficus benjamica*
49		*Ficus callophylla*
50		*Ficus depressa*
51		*Ficus hispida* L.f. var. *hispida*
52		*Ficus microcarpa*
53		*Ficus rumphii*
54		*Ficus sundaica*
55		*Ficus superba* var. *japonica*
56		*Ficus superba* var. *superba*
57		*Ficus tinctoria gibbosa*
58		*Streblus asper*
59		*Streblus ilicifolius*
60	Polypodiaceae	*Drynaria quercifolia*
61	Rhamnaceae	*Ventilago cristata*
62	Rubiaceae	*Pavetta indica*
63	Rutaceae	*Glycosmis pentaphylla*
64	Sapindaceae	*Allophylus glaber*
65		*Dimocarpus longan*
66	Sapotaceae	*Planchonella obovata*
67	Sonneratiaceae	*Sonneratia caseolaris*
68	Sterculiaceae	*Byttneria echinata*
69	Urticaceae	*Poikilospermum suaveolens*
70	Vitaceae	*Ampelocissus hamandii*
71		*Cayratia japonica*
72		*Cayratia trifolia*
73		*Cissus hexangularis*
74		*Tetrastigma quadridens*

Appendix M. Unique sequences (corresponding to Tables 7.4a and b) identified in TSNM samples.

Sequence no.	**Sequences**
1	atcctgtgttcagaaaacaaggttcagaaagcgagaatcaaaaacagaaaaag
2	atcctgttttcagaaaacaagggttcagaaagcgagaaccaaaaaaag
3*	atcctgttttcgaaaataaacaaagattcagaaagcgaaaataaaaaaag
4	atcctgtgttcagaaaacaaggttcagaaagcgagaaccaaaaaaag
5	atcctgttttcagaaaacaagggttcagaaagcgagaatcaaaaacagaaaaag
6	atcctcttttccgaaaacaaaggttcagcaagcgaaaacaagg
7	atcctgttttccgaaaaccagaaaacgctaataaaaaaag
8	atcctgttttccgaaaccaaacaaaggtcagaaagcgaaaaaaaaag
9	atcctgtttttcgaaaacaaacaaagattcagaaagcgaaaataaaaaaag
10	atcctgtgttcagaaaacaaggttcagaaagcgaaaataaaaaaag
11	atctttgttttgagagaaaaaacgatggaaaatgagaataaaaagg
12	atcctgttttccgaaaacaaacaaaggttcagaaggcgaaaaaagg
13	atcctgttttcagaaaacaagggttcagaaagcgagaaccaaaaacagaaaaag
14	atcccgttttccgaaaccaaaggttcagaaagtgaaaaaag
15	atcctgttttctgaaaacaagggttcagaaagcgagaaccaaaaaaag

16	atcctgttttcagaaaacaagggttcagaaagcgaaaataaaaaag
17	atcctgttttccgaaaaccaagaagagttcagaaagggaaaatcaaataaaaaagg
18	atcctgtttttcgaaaacaaaaaaaacaacaaaggttcataaagacagaatcagaataaaaag
19	atcccgttttatgaaaacaaaacaaacaggggttcagaaagtgagaaaagg
20	atcctgttttcgaaaataaacaaagattcagaaagcgagaaccaaaaaaag
21	atcctgttttcagaaaacaagggttcagaaagcgagaaccaaaaaaaaag
22	atccttttttctcaaaacaaaggttcagaaaacgaaaaaaaaag
23	atccttttttccgaaaacaaatcaaataagggcttcgaaagcgagaaaaaaag
24	atcctgttttctgaaaacaaacaaaggttcagaaagcgataataaaataaaaaaggatagatagg
25	atcctgttttccgaaaccaaacaaaggtcagaaagcgaaaataaaaaaag
26	atcctgttttccacaaacaaaggttcataaaacgaaaaaag
27	atccttgttttgagaaaaag
28	atcctgttttccccaaacaagagttcagaaagaaaaaagg
29	atcctgttttccgaaaacaaacaaaggttcataaagacaga
30	atcctgttttattaaaacaaacaagggtttcataaaccgagaataaaaaag
31	atcctgtttttccgaaaacaaaggttcagcaagcgaaaacaagg
32	atcctgtttttgttttccgaaaaaaaaaagattgataaagaaaaaacataaaaaaagaaagaaagaaaacaaaaaaa

*Sequence no. 3 is the same sequence as the one identified in the positive control for *Manilkara zapota* (Sapotaceae).

Appendix N. Food plant taxa of Tonkin snub-nosed monkeys in Vietnam based on field observations from published studies (Boonratana & Le Xuan Canh 1998; Le Khac Quyet *et al.* 2007; Covert *et al.* 2008; Dong Thanh Hai 2011) and diet metabarcoding from current research (only taxa identified to at least species level are listed here).

No.	**Family**	**Species**
1	Aceraceae	*Acer chapaense*
2		*Acer tonkinensis*
3	Anacardiaceae	*Choerospondias axillaris*
4	Annonaceae	*Alphonsea tonkinensis*
5	Apocynaceae	*Melodinus tourneri*
6	Aquifoliaceae	*Ilex macrocarpa*
7		*Ilex purpurea*
8	Araceae	*Rhaphidophora hookeri*
9	Araliaceae	*Brassaiopsis stellata*
10		*Schefflera delavayi*
11		*Schefflera palmiformis*
12		*Schefflera* aff. *velunosa*
13		*Trevesia palmate*
14	Asclepiadaceae	*Goniostemma punctatum*
15	Clusiaceae	*Garcinia bracteata*
16		*Garcinia fagraeoides*
17		*Garcinia tinctoria*
18	Ebenaceae	*Diospyros choboensis*
19		*Diospyros pilosula*
20	Euphorbiaceae	*Sapium rotundifolium*
21	Fabaceae	*Bowringia callicarpa*
22		*Dalbergia tonkinensis*
23	Fagaceae	*Castanopsis chinensis*
24		*Castanopsis tonkinensis*
25		*Quercus acustissima*
26	Gesneriaceae	*Anna submontana*
27	Icacinaceae	*Iodes seguini*
28	Lauraceae	*Beilschmiedia* aff. *vidalli*
29		*Cinnamomum burmannii*
30		*Litsea baviensis*
31		*Machilus bonii*

32	Meliaceae	*Sandoricum kontape*
33		*Toona sinensis*
34	Menispermaceae	*Diplospora viridiflora*
35	Myrsinaceae	*Ardisia crispa*
36		*Ardisia quinquegona*
37		*Ardisia ramondiaeformis*
38	Myrtaceae	*Syzygium wightianum*
39		*Syzygium zeylanicum*
40	Orchidaceae	*Bulbophyllum affine*
41		*Bulbophyllum pectinatum*
42		*Tropidia curculigoides*
43	Passifloraceae	*Adenia heterophylla*
44	Phyllanthaceae	*Bridelia monoica*
45		*Bridelia retusa*
46	Rhamnaceae	*Berchemia floribunda*
47	Rosaceae	*Rubus moluccana*
48	Rubiaceae	*Gardenia sootepesis*
49		*Pavetta tonkinensis*
50	Sabiaceae	*Meliosma fordii*
51	Sapindaceae	*Pometia pinnata*
52	Sapotaceae	*Sarcosperma laurium*
53		*Sinosideroxylon wightianum*
54	Theaceae	*Camellia sasamqua*
55	Tiliaceae	*Excentrodendron tonkinensis*
56	Ulmaceae	*Celtis sinensis*
57	Urticaceae	*Debregeasia squamata* f. *etuberculata*
58	Verbenaceae	*Premna balansae*
59		*Premna flavescens*
60	Vitaceae	*Tetrastigma gaudichaudianum*
61		*Tetrastigma oliviforme*