Diet analysis of Leopoldamys Neilli, a cave-dwelling rodent in Southeast Asia, using next-generation sequencing from feces

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DIET ANALYSIS OF *LEOPOLDAMYS NEILLI*, A CAVE-DWELLING RODENT IN SOUTHEAST ASIA, USING NEXT-GENERATION SEQUENCING FROM FECES

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Abstract: *Leopoldamys neilli* is a Murinae rodent endemic to limestone karst of Thailand and the Lao PDR, but its ecology and the reasons of its endemism to karst are still totally unknown. The aim of this pilot study was to examine the plant composition of the diet of *L. neilli* at the level of order and family using DNA for molecular identification and to compare it with two other forest-dwelling *Leopoldamys* species, *L. herberti* and *L. sabanus*. A 202bp fragment of the *rbc*L gene was amplified and sequenced for twenty-three fecal samples of the three species using 454 pyrosequencing. We successfully identified a total of seventeen orders and twenty-one plant families, corresponding to thirty-three putative species, in the feces of these three *Leopoldamys* species. Solanaceae were the most common plants in the diet of *L.neilli* regardless of the region and sampling season, and they were also present in feces of both *L. herberti* and *L. sabanus*. The Araceae, Fabaceae, and Apocynaceae families were also identified in feces of *L. neilli* collected in various regions of Thailand and at different seasons. Plants of the Oleaceae family are consumed by both *L. herberti* and *L. sabanus* but were not found in the diet of *L. neilli*. Further improvements of the study, such as the use of additional genes, the creation of a reference collection, the microhistological examination of plant fragments to determine which parts of the plant are consumed, and the analysis of the animal diet of *Leopoldamys* are suggested to enhance the quality and accuracy of the results obtained.

Introduction

Several Murinae rodents endemic to limestone karst have been described in Southeast Asia, but their ecology is still poorly known. *Niviventer hinpoon* (Marshall, 1977) is found in Thailand, *Saxatilomys paulinae* (Musser et al., 2005) in the Lao PDR, and *Tonkinomys daovantieni* (Musser et al., 2006) in Vietnam, while *Leopoldamys neilli* (Marshall, 1977) has been described in Thailand but has also recently been discovered in the Lao PDR (Balakirev et al., 2013; Latinne et al., 2013a). Recent phylogeographic studies of *L. neilli* revealed a deep genealogical divergence among geographically close lineages of this species in Thailand and a high population fragmentation related to the patchy distribution of limestone karst (Latinne et al., 2011; Latinne et al., 2012). Such strong phylogeographic structure is not observed for other Murinae rodents in Thailand that are characterized by lower habitat specialization (Latinne, 2012). These results suggested that the spatial isolation of karst areas prevents migration among lineages of *L. neilli* and indicated a close association of this species with this habitat. However, ecological data on *L. neilli* are lacking, and the reasons of its endemism to limestone karst are still totally unknown. A better knowledge of the ecology of *L. neilli*, notably its feeding habits, is thus necessary for determining if diet contributes to the habitat specialization and distributional limits of this species, as well as for understanding its functional role in karst ecosystems.

Rodents and other small mammals living in forests of Southeast Asia are generally considered to be omnivorous (Emmons, 2000; Langham, 1983; Lim, 1970), and they play a key role in the food chain, both as consumers of plants and small invertebrates, and as food resources for larger predators. Rodents may also play an important role in the frugivores' community as seed dispersers or seed predators, and it has been suggested that some *Leopoldamys* species might benefit seed recruitment of several tree species by seed hoarding or seed ingestion in Southeast Asia and China (Cheng et al., 2005; Wells et al., 2009; Zhang et al., 2008). However detailed information on the exact diet composition of Southeast Asian rodents remains scarce and should be improved to better understand the trophic

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relationships in Southeast Asian ecosystems and the functional role of rodents in these biological communities, as well as the resource partitioning among competing species.

Direct observations of foraging and feeding behaviors are generally time-consuming, and they are particularly difficult to obtain for small nocturnal mammals living in karst habitats. Feces analysis represents an efficient and non-invasive alternative to circumvent this problem. Microhistological examination of plant and invertebrate fragments in fecal samples has been traditionally used, but this method requires a lot of time and training, and its results are often imprecise (Soininen et al., 2009; Emmons, 2000). More recently, molecular techniques using DNA barcoding have been developed to successfully analyze the diet of wild herbivores from feces (Bradley et al., 2007; Kim et al., 2011; Soininen et al., 2009; Valentini et al., 2009). These methods aim to amplify small, but highly variable, DNA fragments contained in the feces with universal primers and use them as barcodes to identify the plant taxa that have been eaten. Several DNA regions have been used for this purpose in the literature, and the choice of the target segment results from a compromise among a minimal size, a maximal genetic distance between species, a minimal genetic diversity within species, and the existence of an adequate reference collection (Bradley et al., 2007). As feces contain only highly degraded DNA, the length of fragments that can be amplified is usually shorter than 200 base pairs (bp).

Using a 202bp short segment of the ribulose-bisphosphate carboxylase (*rbc*L) gene of the chloroplast genome as a barcode region, the present study was designed as a pilot study to assess the performance of this method in analyzing the plant composition of the diet of *L. neilli* at the level of order and family. Another objective of this study was to compare the diet of *L. neilli* with two other forest-dwelling *Leopoldamys* species also found in Thailand but non-endemic to limestone karst, *L. sabanus* and *L. herberti.* (*L. herberti* was previously thought to belong to *L. edwardsi*, but several recent studies have shown that it should be regarded as a distinct species from *L. edwardsi* (Balakirev et al., 2013; Latinne et al., 2013a).

Methods

Twenty-six fecal samples from the three *Leopoldamys* species were collected from nineteen localities (Fig. 1) below traps, baited with ripe banana, where the animals were caught during a survey of the rodent diversity in Thai limestone karst. The samples were preserved in silica gel. Two mitochondrial genes were sequenced for all trapped animals using tissue biopsy from the ear to reliably identify them at the species level (Latinne et al., 2013b). The specific status of these individuals was also confirmed by an independent molecular analysis using a mitochondrial mini-barcode from feces (Galan et al., 2012). DNA was extracted from feces using the QIAamp DNA Stool Kit (Qiagen) and following the protocol designed for the isolation of DNA from human stool.

A 202bp fragment of the *rbc*L gene was amplified for each sample using universal primers Z1aF and hp2R (Hofreiter et al., 2000), modified by the addition of a specific tag on the 5′ end, following the tagging and multiplexing method for the 454 pyrosequencing developed by Galan et al. (2010). This tag consists of a short 7bp sequence to allow the recognition of the sequences after the pyrosequencing where all the PCR products from the different samples are mixed together and a 30bp Titanium adaptor required for the emPCR and 454 GS-FLX pyrosequencing using Lib-L Titanium Series reagents. Six and five different tags were designed for the forward and the reverse primers, respectively. This gives thirty putatively unique combinations of forward and reverse tags, and thus, allows tagging up to thirty different amplicons.

PCRs were carried out in a 10 µL reaction volume using 5 µL of 2x QIAGEN Multiplex Kit (Qiagen), 0.5 µM of each primer, and 2 µl of DNA extract. The PCR started by an initial denaturation step at 95 °C for 15 min, followed by forty cycles of denaturation at 94 °C for 30 s, annealing at 45 °C for 45 s, and extension at 72 °C for 30 s, followed by a final extension step at 72 °C for 10 min.

Positive PCR products were then pooled together for 454 pyrosequencing using 3 µL per strong PCR amplification products or 7 µL per lighter ones. The PCR pool was processed by Beckman Coulter Genomics (Danvers, Massachusetts). Amplicons were sequenced after the emPCR on a 454 Genome Sequencer FLX (Roche) in 1/4th of titanium picotiter plate.

The software SESAME 1.1B (Meglécz et al., 2011) was used to sort the sequences. Thanks to the tag combinations, the sequences were assigned to the fecal sample from which the PCR amplicon was obtained. Artifactual variants due to sequencing errors during PCR, emPCR, and 454 sequencing were discarded as described in Galan et al. (2012).

The validated variants of *rbc*L sequences obtained were compared with published *rbc*L sequences available on GenBank using NCBI's BLASTN program (Zhang et al., 2000) and were assigned to order and family of the closest sequences (with at least 98% of identity and 100% of query coverage) following the APG III classification (Bremer et al., 2009).

Results

Out of the 26 *Leopoldamys* feces analyzed in this study, 23 were successfully amplified (Table 1) and a total of 392 *rbc*L sequences, including 112 distinct variants corresponding to 33 validated variants, were obtained with a mean of 15 sequences per samples. Each variant was assigned unambiguously to one plant family, with the exception of four sequences of the Zingiberales order that could belong

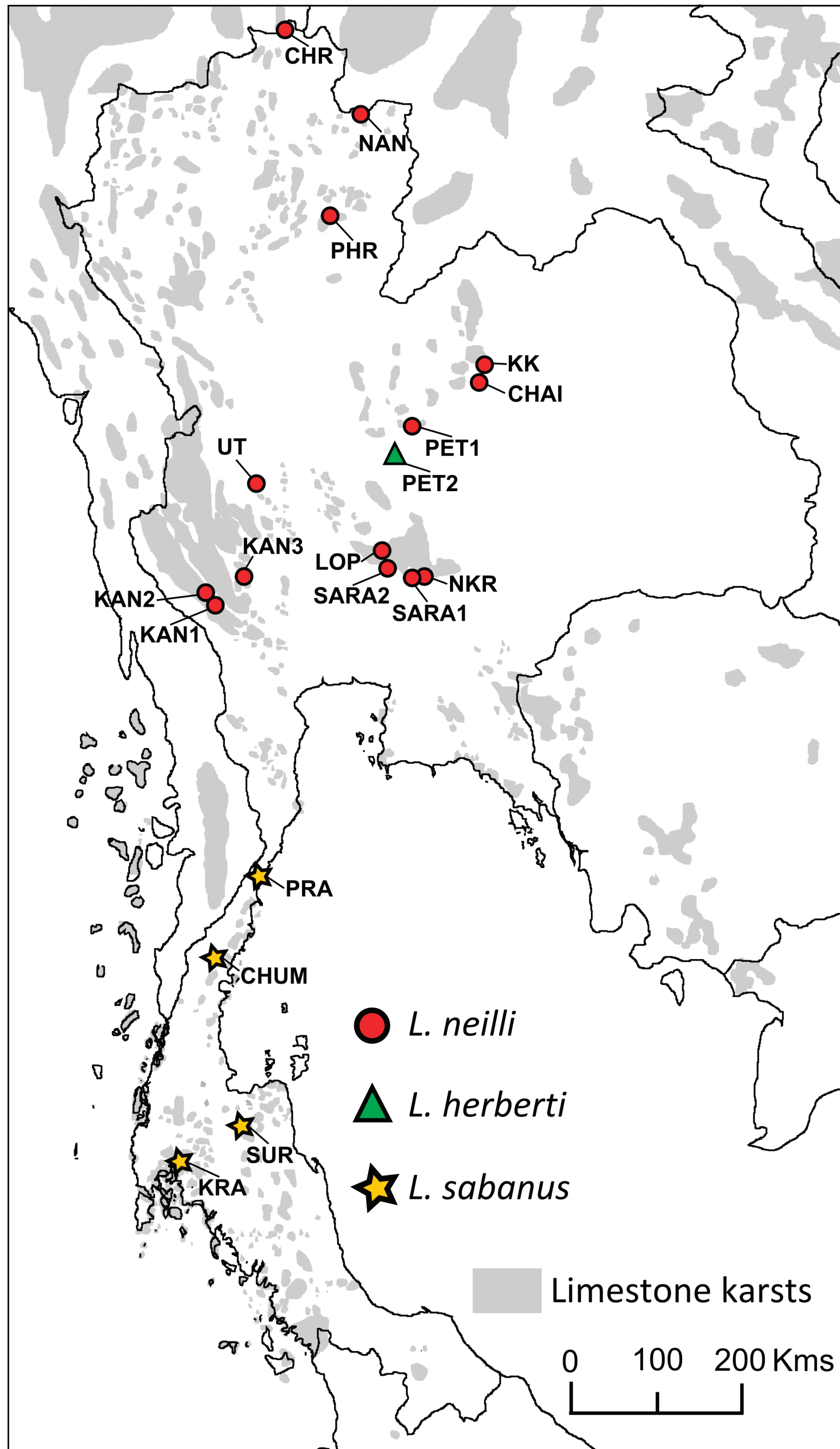

Figure 1. Locations of *Leopoldamys* fecal samples analyzed in this study. The province abbreviations are spelled out in Table 1.

Table 1. Sample locations, regions, and seasons for the *Leopoldamys* fecal samples in this study. See Figure 1 for a map of the locations.

Species	Sample ID	Province (Locality)	Region	Season	PCR Success
Leopoldamys neilli	F161	Kanchanaburi (KAN1)	West	Dry	Yes
	F567	Kanchanaburi (KAN1)	West	Rainy	Yes
	F172	Kanchanaburi (KAN2)	West	Dry	Yes
	F565	Kanchanaburi (KAN2)	West	Rainy	Yes
	F191	Kanchanaburi (KAN3)	West	Dry	Yes
	F577	Kanchanaburi (KAN3)	West	Rainy	Yes
	F554	Uthai Thani (UT)	West	Rainy	Yes
	F313	Chaiyaphum (CHAI)	Northeast	Dry	Yes
	F327	Khon Kaen (KK)	Northeast	Dry	Yes
	F331	Khon Kaen (KK)	Northeast	Dry	Yes
	F418	Petchabun (PET1)	Northeast	Dry	Yes
	F399	Nan (NAN)	North	Dry	Weak
	F406	Nan (NAN)	North	Dry	Weak
	F391	Phrae (PHR)	North	Dry	Yes
	F441	Chiang Rai (CHR)	North	Dry	Yes
	F505	Saraburi (SARA1)	Centre	Rainy	Weak
	F508	Nakhon Ratchasima (NKR)	Centre	Rainy	Weak
	F534	Saraburi (SARA2)	Centre	Rainy	Weak
	F538	Lopburi (LOP)	Centre	Rainy	Yes
Leopoldamys herberti	F420	Petchabun (PET2)	Northeast	Dry	Yes
	F424	Petchabun (PET2)	Northeast	Dry	No
	F430	Petchabun (PET2)	Northeast	Dry	Yes
Leopoldamys sabanus	F254	Krabi (KRA)	South	Rainy	Yes
	F298	Surat Thani (SUR)	South	Rainy	No
	F445	Prachuap Khiri Khan (PRA)	South	Rainy	Yes
	F477	Chumphon (CHUM)	South	Rainy	No

either to Marantaceae or Musaceae families (Table 2). Several *rbc*L variants were assigned to the same family and could represent different plant species if each variant belongs to a different species, but this assumption should be confirmed by further analyses. A total of 17 orders and 21 plant families, corresponding to 33 putative species, were identified in the feces of the *Leopoldamys* species (Table 2).

The diet of *Leopoldamys neilli* is quite diversified, with seventeen orders and nineteen families identified within feces of this species. Plants belonging to Solanaceae (corresponding to a single validated variant) and Marantaceae/Musaceae (corresponding to four validated variants) were identified in ten out of the nineteen feces of *L. neilli* analyzed (53%). Solanaceae are also identified in the two feces of *L. sabanus* (100%) and one of the two feces of *L. herberti* (50%). Plants of the Oleaceae family are consumed by both *L. herberti* and *L. sabanus* but were not found to be consumed by *L. neilli*.

Solanaceae and Marantaceae/Musaceae were highly common in the diet of *Leopoldamys neilli* as they were eaten by specimens in all sampled regions (Fig. 2). Most of the plant families identified in this study (14/19) were encountered in only one *L. neilli* sample, but the Araceae, Fabaceae, and Apocynaceae families were identified in samples collected in various regions of Thailand and at different seasons.

Discussion

This pilot study is the first study of the diet composition of *Leopoldamys neilli* that remained totally unknown up to now. We successfully identified a total of seventeen orders and nineteen plant families, corresponding to thirty putative species, in the feces of this long-tailed giant rat endemic to limestone karst of Thailand and the Lao PDR. The plant diversity observed in the *L. neilli* feces is high and similar to the one described for large herbivores species using similar methods of molecular identification (Bradley et al., 2007; Kim et al., 2011; Valentini et al., 2009). Plants identified in the diet of *Leopoldamys* species are all flowering plants (angiosperms), and most of these plant families have been observed in the flora of limestone karst in southern Vietnam (International Finance Corporation, 2002). Even though a recent study showed that the primers Z1aF and hp2R used in our study also allow the amplification of sequences belonging to ferns or mosses (Kim et al., 2011), no fern or moss was detected in the feces of the three studied *Leopoldamys* species.

Table 2. Plant families identified in the feces of three *Leopoldamys* species in Thailand, with number and frequency of occurrence.

Order	Family	Number of Validated Variants	Frequency		
			L. neilli (n = 19)	*L. herberti* (n = 2)	*L. sabanus* (n = 2)
Alismatales	Araceae	2	3 (16%)		
Brassicales	Brassicaceae	1	1 (5%)		
Commelinales	Commelinaceae	1	1 (5%)		
Cucurbitales	Cucurbitaceae	1	1 (5%)		
Dioscoreales	Dioscoreaceae	1	1 (5%)		
Fabales	Fabaceae	5	4 (21%)		
Fagales	Fagaceae	1	1 (5%)		
Gentianales	Apocynaceae	3	3 (16%)		
Lamiales	Lamiaceae	1	1 (5%)		
	Oleaceae	2		1 (50%)	1 (50%)
Malpighiales	Phyllanthaceae	1	1 (5%)		
	Putranjivaceae	1			1 (50%)
Malvales	Malvaceae	1	1 (5%)		
Poales	Poaceae	1	1 (5%)		
Piperales	Aristolochiaceae	1	1 (5%)		
Rosales	Rhamnaceae	1	1 (5%)		
Sapindales	Burseraceae	1	1 (5%)		
	Sapindaceae	2	1 (5%)		1 (50%)
Solanales	Convolvulaceae	1	2 (10%)		
	Solanaceae	1	10 (53%)	1 (50%)	2 (100%)
Zingiberales	Marantaceae or Musaceae[a]	4	10 (53%)	2 (100%)	

[a] Possible contamination by the bait.

Species of the Solanaceae and Marantaceae/Musaceae families are the most common plants identified in the diet of *Leopoldamys neilli* regardless of the region and season. However, traps used in this study were baited with ripe banana (*Musa* sp., a genus of the Musaceae family), and these bananas were probably eaten by trapped rats several hours before the collection of feces, because the feces were collected at least twelve hours after trap setup. Direct contact of fecal samples with banana was also possible in the trap. The frequent presence of Musaceae in the feces of *L. neilli* could thus represent a bias due to the bait used, rather than the real diet of this species. Therefore the Marantaceae/Musaceae families should not be included positively in the diet of *L. neilli* without further verification.

As the number of fecal samples analyzed successfully for *Leopoldamys herberti* and *L. sabanus* was much lower than for *L. neilli*, it is not possible to compare rigorously the diet composition of these three species. Despite the small number of samples, Solanaceae were also identified in feces of both *L. herberti* and *L. sabanus*. Therefore this plant family seems to be very common in the diet of all the *Leopoldamys* species in Thailand. Solanaceae are represented in Southeast Asia by the Solanoideae subfamily and may take the form of herbs, shrubs, or small trees in this region, but the lack of resolution at the species level of the *rbc*L fragment that we used does not allow us to get more information on the type of Solanaceae consumed by the *Leopoldamys* species.

CONCLUSION

Despite the limitations and small sample size of this pilot study, these preliminary results confirm that DNA barcoding from feces is a promising tool to better understand the feeding habits of *Leopoldamys neilli*. We suggest some improvements for future studies to enhance the quality and accuracy of the results.

First, a better knowledge of the flora of Thai limestone karst is absolutely needed to allow plant identification at lower taxonomic level than order and family. The creation of a reference collection by sampling, identification, and DNA sequencing of the most common plants of Thai limestone karst would help to assess more accurately the diet of these species and allow more precise identifications of the sequences obtained from feces than data now in public databases such as GenBank (Valentini et al., 2009). We also suggest using other highly variable DNA regions such as *trnH, psbA* (Kress and Erickson, 2007), *matK* (Hollingsworth et al., 2009), *trn*L (Taberlet et al., 2007; Valentini et al., 2009), or *ITS-2* (Bradley et al., 2007) as DNA barcodes in association with *rbc*L to obtain more

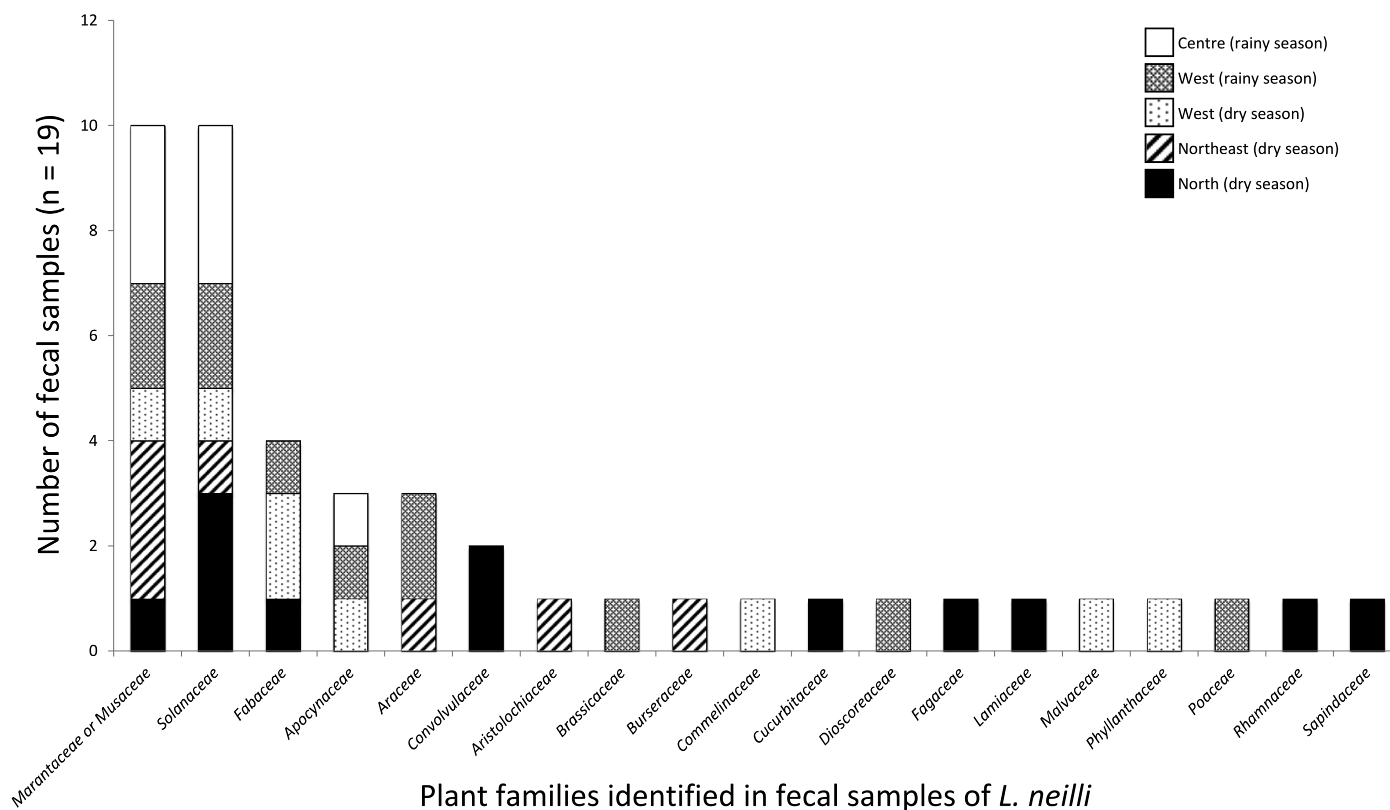

Figure 2. Numbers of samples of feces of *Leopoldamys neilli* out of total of nineteen showing plant families, with samples coded for season and region.

precise results. Checking traps for captures more frequently and collecting feces more rapidly after trap setup would prevent bait contamination of the feces. The use of different baits or baits distinct from all plant species known to occur in the studied region will also help to determine whether Musaceae is part of the natural diet of *Leopoldamys neilli* or not.

Moreover, combining DNA-based analysis of feces with microhistological examination of plant fragments in fecal samples would help to determine which parts of the plant are consumed by *L. neilli* and other *Leopoldamys* species, as this information remains unknown when using DNA barcoding. In particular, the study of the diversity, quantity, and viability of seeds defecated by these long-tailed giant rats is needed to better assess their potential role as seed dispersers in Southeast Asian ecosystems via seed ingestion and subsequent defecation, as already suggested for *L. sabanus* by Wells et al. (2009).

It could also be very interesting to perform such DNA barcoding analysis using universal primers designed to amplify animal DNA, because the *Leopoldamys* species also eat small preys such as insects or snails (Langham, 1983; Lim, 1970). A small fragment of the cytochrome *c* oxidase I gene (Hajibabaei et al., 2011) could be the ideal marker for this purpose.

Finally, most of the plant families identified within our dataset were encountered in only one sample. This observation strengthens the importance of studying a large number of samples to obtain an exhaustive list of the plant composition of the *Leopoldamys* diet to better comprehend the whole diversity of food resources consumed by these long-tailed giant rats and how it may vary in space and time and among species.

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