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New data on trophic associations of dung beetles (Coleoptera, Scarabaeidae, Scarabaeinae) and lemurs (Primates, Lemuroidea) in Madagascar

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Abstract

Resource use and diet specialization of Madagascan dung beetles has been little studied especially concerning the possible associations between specific dung beetle and lemur species. Pilot studies have demonstrated that amplicon metagenomics is a promising tool for the lemur inventories. In the present contribution, we report new results of the gut content analysis of three endemic Madagascan dung beetles species: *Helictopleurus clouei* (Harold), *Epilissus apotolamproides* (Lebis) and *Nanos dubitatus* (Lebis). Amplicon metagenomics revealed trophic associations of these species with *Eulemur sanfordi* (Archbold), *Eu. fulvus* (É.Geoffroy Saint-Hilaire), and *Cheirogaleus crossleyi* (Grandidier), respectively. The reads of other mammal species, revealed by the analysis, including putative contaminations, are discussed.

Keywords

Scarabaeines, coprophagy, gut content analysis, food chains, next-generation sequencing, amplicon metagenomics

Introduction

Madagascar has long been recognized for its exceptional biodiversity and high level of endemism, yet it has experienced severe primary vegetation lost due to the anthropogenic pressure. Since the concept of biodiversity hotspots was introduced, Madagascar was ranked first in terms of the five most important factors, including endemic taxa per area ratio and percentage of remaining primary vegetation (Myers et al. 2000). Dung beetles (Scarabaeidae, Scarabaeinae) are important elements in the food webs of ecosystems in Madagascar, where they originally evolved as consumers of lemur excrements (Wirta and

Montreuil 2008, Wirta et al. 2008, Viljanen et al. 2010b, Wirta et al. 2010). The increased loss of primary forests, where the majority of dung beetles live, as well as introduction of non-native mammals, may result in significantly rearranged tropical food chains (Hanski et al. 2008, Rahagalala et al. 2009, Wirta et al. 2014).

The study of the dung beetle trophic associations and feeding behavior was so far mostly based on field surveys with standardized trapping protocols (Raine and Slade 2019). The development of the new generation sequencing (NGS) provides new possibility to examine the diet of beetles by sequencing multiple DNA copies extracted from their gut content. A few pilot studies demonstrated the usage of amplicon metagenomics to investigate the food sources for dung beetles (Gómez and Kolokotronis 2017, Kerley et al. 2018, Drinkwater et al. 2021, Frolov et al. 2023). The amplicon metagenomic method was shown to be a promising tool for the lemur inventories in Madagascar (Frolov et al. 2023). It will also allow evaluating dung beetle capacity to shift towards other food resources and, thus, providing a better understanding of the consequences of the biodiversity crisis in Madagascar. However, the trophic associations of different dung beetle species and their food producers are still poorly known. The present paper is a contribution to our better understanding of the trophic associations of native Madagascan dung beetles based on amplicon metagenomic analysis of the gut content of three species.

Material and methods

Sampling localities, material, and collecting methods

Beetles were collected in two localities in central and northern Madagascar. The localities are described in detail by Akhmetova et al. (2023). Voucher specimens are housed in the collection of Zoological Institute, Saint Petersburg, Russia (ZIN) and National Museum of Natural History, Paris, France (MNHN).

Helictopleurus clouei (Harold 1869)

Madagascar • Antsiranana: Ankarana Special Reserve, open area near Manongarivo, 12°51'55"S 49°13'24"E, cow dung, 6.II.2022, A.V.Frolov leg., one female (ZIN).

Epilissus apotolamproides (Lebis, 1961)

Madagascar • Toamasina: Analamazaotra Special Reserve, primary forest, 18°55'59"S 48°25'12"E, pitfall traps baited with human feces, 17-20.II.2022, A.V.Frolov leg., one female (ZIN).

Nanos dubitatus (Lebis, 1953)

Madagascar • Toamasina: Analamazaotra Special Reserve, primary forest, 18°55'59"S 48°25'12"E, pitfall traps baited with human feces, 17-20.II.2022, A.V.Frolov leg., one male (MNHN), two females (ZIN).

The beetles were collected from cow dung and by pitfall traps baited with human feces. A pitfall trap was a 1-liter plastic container 10 cm in diameter buried in soil. A bait was placed in a 5 cm diameter cup wrapped in gauze and suspended by a wire above a collecting container. To avoid flooding, the traps were covered with plastic lids attached by wooden sticks about 4 cm above the ground. Funnels were placed over the collecting jars, so the beetles attracted to the traps fell into the jars and stayed alive until retrieval. The traps were exposed overnight. After retrieval, the beetles were placed in containers with 96% ethanol and transported to the laboratory after two or three weeks at room temperature; the alcohol was changed twice. The hindguts were dissected under a stereomicroscope and placed in Eppendorf tubes with 96% ethanol.

DNA extraction and sequencing

DNA was extracted by phenol-chloroform method (Sambrook and Russell 2006). The extracted DNA was quantified using a Qubit fluorimeter 4.0 with high-sensitivity reagents (Lumiprobe QuDye dsDNA HS Assay Kit) and 1 µl of DNA. Three samples with the highest DNA concentration were used for high throughput sequencing along with a control sample (distilled water). For amplicon metagenomic sequencing the following primer pair was used: 16Smam1 (5'-CGGTTGGGGTGACCTCGGA-3') and 16Smam2 (5'-GCTGTTATCCCTAGGGTAACT-3'). These primers amplify a short (90–95 bp) yet informative region of lrRNA and were designed to be specific for mammals (Taylor 1996). They were successfully used in a few recent works (Drinkwater et al. 2021, Ji et al. 2022, Frolov et al. 2023). NGS libraries were prepared using the NEBNext Ultra II DNA Library Prep Kit, checked with Qubit (high-sensitive reagents) and real-time PCR for quantification, and Bioanalyzer for size distribution detection. The amplicon paired-end libraries (PE250) targeting an insert size of 350 bp were sequenced on Illumina NovaSeq 6000 platform aiming for 30K raw tags per sample. DNA extraction was performed at Chromas Core Facility, Saint Petersburg State University (Peterhoff, Russia), Research Park, and library preparation, quality control, and sequencing were performed at Novogene (Cambridge, UK).

Bioinformatics methods

Demultiplexed raw paired reads were merged and quality filtered with usearch v11 software (Edgar and Flyvbjerg 2015). Primers were trimmed and the reads were quality filtered with -fastq_maxee 1.0 option. The reads were relabeled to add sample identifiers and pooled to enhance sensitivity of analysis. OTU (operational taxonomic unit) analysis was carried out with two approaches implemented by usearch v11: UPARSE (generating OTUs by clustering reads with 97% similarity) (Edgar 2013) and UNOISE (generating ZOTUs based on error-correction) (Edgar and Flyvbjerg 2015) with -minsize 11 option. Both methods produced similar results, with total number of OTUs being slightly smaller than ZOTUs, therefore OTUs were used for downstream analysis. OTUs were then manually annotated with BLAST (https://blast.ncbi.nlm.nih.gov) using megablast algorithm against nucleotide database.

Results

Illumina sequencing yielded 247816 reads for three samples. After merging and quality filtering, we obtained 205762 clean reads (over 83% of raw reads). Analysis with usearch revealed 48 OTUs and 52 ZOTUs. Statistics per sample are given in Table 1.

After annotation of OTUs with BLAST, 14 of them returned no hits, 17 ZOTUs were annotated as human pseudogenes (numts), and five OTUs were attributed to non-mammalian species. After these OTUs were discarded, 12 OTUs were retained which comprised 193069 reads. They belonged to eleven mammal species. The reads resulted from possible contaminations are discussed below and are not included in Table 2.

Discussion

Resource use or diet specialization of Madagascan dung beetles has been little studied especially concerning the possible associations between specific dung beetle and lemur species. Viljanen (2004) conducted the first study on the topic in Ranomafana National Park by using lemur feces baited pitfall traps to attract dung beetles. The park is located within the eastern wet forest belt in SE Madagascar. The study included feces of seven lemur species that occur in the park (omnivorous *Microcebus rufus* (Geoffroy), frugivorous *Eulemur rubriventer* (Geoffroy), vegetarian large bodied *Propithecus edwardsi* Grandidier and three bamboo eating species that belong to the genus *Hapalemur* Geoffroy at that time). In addition to lemur feces baited traps, fish, meat of pig and zebu-cattle, rotten fruit and cattle dung baited traps were used. However, no clear associations of specific dung beetle and lemur species were found. To date, the only known six individuals of *Epilissus genieri* Montreuil were trapped solely with the *Hapalemur aureus* (Meier) feces bated traps. However, due to trapping design this result maybe an artefact. In addition, small-bodied *Helictopleurus semivirens* Lebis seemed to prefer the feces of the largest bodied lemur species *P. edwardsi*. In Madagascar, general pattern seems to be that a half of wet forest inhabiting endemic dung beetle species are either carrion feeders or generalist feeding on both carrion and feces, and the other half might be considered dung specialists excluding cattle dung (Viljanen et al. 2010b). Dung specialist species seem to prefer feces of different lemur species in relation to their size and diet – large-bodied vegetarian or frugivorous species were preferred over bamboo eating or omnivorous species (Viljanen 2004). However, most of the dung specialist species are easily collected with human feces baited traps. Human feces most likely act as a substitute of the feces of extinct large-bodied lemur species. Indeed altogether 46 dung beetle species of know 285 species have been trapped with human feces bated traps in Madagascar (Viljanen, unpublished data). The totally new type of resource, cattle dung, is not used by wet forest dwelling dung beetle species, but interestingly several endemic western dry forest species have switched their habitat and resource use from forest and carrion or lemur feces to open areas and cattle dung within the last 1,500 years. These species have been able spread across the island (Hanski et al. 2008, Rahagalala et al. 2009). In comparison, wet forest species have much more limited

ranges along the eastern wet forest belt species turnover (beta-diversity) being extremely high compared to other tropical areas (Viljanen et al. 2010a).

In the pilot study using amplicon metagenomic analysis of Madagascan dung beetles, Frolov et al. (2023) provided evidence of trophic association of four dung beetle species and six lemur species. The present results are congruent with the previous work. The majority of reads from two samples of the three belonged to human and cow. Both food sources are available in the sampling areas. The exception is *H. clouei* specimen, where over 40% of total reads belonged to small Indian civet (*Viverricula indica* Geoffroy). The civet DNA was also found in *E. apotolamproides* sample, although in a smaller number. Small Indian civet is non-native to Madagascar, but is now widely distributed on the island (Shehzad et al. 2012).

The reads of house mouse (*Mus musculus* Linnaeus) and pig (*Sus scrofa* Linnaeus) may be a result of contamination or indicate a real, though probably occasional, feeding on the feces of these animals. It was shown that the DNA of domestic and synanthropic animals could be present in PCR reagents (Leonard et al. 2007). On the other hand, these non-native animals are now widely distributed in Madagascar. The *N. dubitatus* sample yielded reads of Norway lemming (*Lemmus lemmus* (Linnaeus)), and *H. clouei* and *E. apotolamproides* yielded reads of Bay duiker (*Cephalophus dorsalis* Gray). Both species do not occur in Madagascar and their reads apparently resulted from contamination. Cat (*Felis catus* Linnaeus) is now widespread in Madagascar but feeding on feces of carnivorous animals was not registered, and considering small number or cat reads with think, that they also resulted from contamination.

Two lemur species were revealed by the annotation of the OTUs with Genbank database: brown lemur (*Eulemur fulvus* (Geoffroy)) and furry-eared dwarf lemur (*Cheirogaleus crossleyi* Grandidier). First species DNA was found in the gut content of *E. apotolamproides* and *H. clouei*, and second – in *N. dubitatus*. Brown lemur was considered a complex of 6–7 subspecies which were elevated to full species by Groves (2006). Brown lemur has a disjunct range with northern part being a small area south of Beramanja (Mittermeier et al. 2008), which is some 70 km south-west of the collecting locality of *H. clouei*. Brown lemur is replaced with Sanford's lemur in the northernmost Madagascar. Wilson et al. (1988) wrote that Sanford's lemur (treated a subspecies of brown lemur) was present in substantial numbers at Ankarana and favored degraded forest or forest bordering the savannah surrounding the massif. This agrees well with the biotope where *H. clouei* was collected. The data available in Genbank (checked 27.11.2023) do not allow differentiating the two species based on the amplified fragment, probably due to the close relations of the taxa. But considering the well studied distribution of lemurs, we consider that the reads from *H. clouei* belong to Sanford's lemur and those from *E. apotolamproides* belong to brown lemur. Furry-eared dwarf lemur is distributed in central-east Madagascar, mostly in the rainforest belt from Andasibe-Mantadia National Park in the south to Zahamena National Park in the north (Lei et al. 2014). Therefore, the collecting localities of sample specimens agree well with known distribution of lemurs.

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Nanopublications

Nanopublication	Creator	Date
Epilissus splendidus Fairmaire, 1889 (species) - trophically interacts with - Propithecus diadema Bennett, 1832 (species)	Andrey Frolov	02-10-2023 11:05:52
Epilissus splendidus Fairmaire, 1889 (species) - trophically interacts with - Hapalemur griseus (Link, 1795) (species)	Andrey Frolov	02-10-2023 10:52:00
Helictopleurus fissicollis (Fairmaire, 1895) (species) - trophically interacts with - Eulemur sanfordi Archbold, 1932 (species)	Andrey Frolov	02-10-2023 10:19:11
Nanos agaboides (Boucomont, 1937) (species) - trophically interacts with - Eulemur sanfordi Archbold, 1932 (species)	Andrey Frolov	02-10-2023 10:16:50
Epilissus splendidus Fairmaire, 1889 (species) - trophically interacts with - Eulemur rubriventer (I.Geoffroy Saint-Hilaire, 1850) (species)	Andrey Frolov	02-10-2023 10:13:01
Helictopleurus fissicollis (Fairmaire, 1895) (species) - trophically interacts with - Eulemur rubriventer (I.Geoffroy Saint-Hilaire, 1850) (species)	Andrey Frolov	02-10-2023 10:09:36
Helictopleurus giganteus (Harold, 1869) (species) - trophically interacts with - Eulemur fulvus (É.Geoffroy Saint-Hilaire, 1796) (species)	Andrey Frolov	02-10-2023 10:06:51
Epilissus splendidus Fairmaire, 1889 (species) - trophically interacts with - Eulemur fulvus (É.Geoffroy Saint-Hilaire, 1796) (species)	Andrey Frolov	02-10-2023 10:03:32
Helictopleurus fissicollis (Fairmaire, 1895) (species) - trophically interacts with - Eulemur coronatus (Gray, 1842) (species)	Andrey Frolov	02-10-2023 09:58:31
Helictopleurus clouei (Harold, 1869) (species) - trophically interacts with - Eulemur sanfordi Archbold, 1932 (species)	Andrey Frolov	30-11-2023 02:34:42
Epilissus apotolamproides (Lebis, 1961) (species) - trophically interacts with - Eulemur fulvus (É.Geoffroy Saint-Hilaire, 1796) (species)	Andrey Frolov	30-11-2023 02:36:50
Nanos dubitatus (Lebis, 1953) (species) - trophically interacts with - Cheirogaleus crossleyi A.Grandidier, 1870 (species)	Andrey Frolov	30-11-2023 02:38:34

Epilissus apotolamproides (Lebis, 1961) (species) - trophically interacts with - Viverricula indica (Desmarest, 1804) (species)	Andrey Frolov	30-11-2023 02:40:17

Conflicts of interest

The authors have declared that no competing interests exist.
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Table 1.

Statistics and bioinformatics results of amplicon metagenomics analysis of gut content of *H. clouei*, *E. apotolamproides* and *N. dubitatus*.

Sample	***H. clouei***	***E. apotolamproides***	***N. dubitatus***
raw reads	92824	89540	65452
merged reads	75383	88362	42050
clean reads	75373	88345	42044
OTUs	37	32	21
ZOTUs	38	33	20

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Table 2.

Results of OTUs annotation with BLAST (excluding contaminations; see Discussion regarding *Eu. sanfordi*). Data are given as a number (#) and percent (%) of reads per sample.

Mammal species	**Sample (beetle specimen)**					
	H . clouei		***E. apotolamproides***		***N. dubitatus***	
	#	%	#	%	#	%
Homo sapiens	13362	19	60421	74	11352	30
Bos taurus	18495	26	17766	22	24718	66
Viverricula indica	30767	43	695	1	0	0
Mus musculus	8093	11	163	0	0	0
Eulemur fulvus	0	0	2752	3	0	0
Eulemur sanfordi	60	0	0	0	0	0
Cheirogaleus crossleyi	0	0	0	0	1059	3
Sus scrofa	702	1	4	0	522	1
Total	71479	100	81801	100	37651	100