Proceedings of the Zoological Institute RAS
Vol. 327, No. 4, 2023, pp. 719–723
10.31610/trudyzin/2023.327.4.719

UDC 595.764.1+577.212.3

Diet shift in giant Madagascan dung beetle *Helictopleurus giganteus* (Coleoptera: Scarabaeidae: Scarabaeinae) studied by amplicon metagenomics

A.V. Frolov[1]*, M.S. Vishnevskaya[1, 2] and L.A. Akhmetova[1]

[1] *Zoological Institute, Russian Academy of Sciences, Universitetskaya Emb. 1, 199034 Saint Petersburg, Russia; e-mail: afrolov@zin.ru*

[2] *Department of Entomology, Saint Petersburg University, Universitetskaya Emb. 7/9, 199034 Saint Petersburg, Russia.*

Submitted November 9, 2023; revised November 23, 2023; accepted December 2, 2023.

ABSTRACT

Dung beetles are important elements in the food webs in Madagascar, where they evolved as consumers of lemur excrements. The anthropogenic pressure reduces lemur populations, which causes dung beetles to shift to other food sources. To assess the diet of giant Madagascan dung beetle *Helictopleurus giganteus* (Harold), we studied hindgut content of seven specimens from different localities with amplicon metagenomic methods. We found reads of five mammal species, with over 99% of total reads belonging to human and cow. No native Madagascan mammals were detected in the samples. The results suggest the human mediated diet shift in *H. giganteus*, although they should be interpreted with caution, because unavoidable contaminations may contribute reasonably to the high yield of the cow and human reads.

Key words: amplicon metagenomics, analysis, coprophagy, Dung beetles, gut content, Madagascar, next-generation sequencing, scarabaeines

Изучение изменения питания гигантского мадагаскарского жука-навозника *Helictopleurus giganteus* (Coleoptera: Scarabaeidae: Scarabaeinae) с помощью ампликонной метагеномики

А.В. Фролов[1]*, М.С. Вишневская[1, 2] и Л.А. Ахметова[1]

[1] *Зоологический институт РАН, Университетская наб. 1, 199034 Санкт-Петербург, Россия; e-mail: afrolov@zin.ru*

[2] *Кафедра энтомологии, Санкт-Петербургский государственный университет, Университетская наб. 7/9, 199034 Санкт-Петербург, Россия.*

Представлена 9 ноября 2023; после доработки 23 ноября 2023; принята 2 декабря 2023.

РЕЗЮМЕ

Жуки-навозники являются важными элементами пищевых сетей на Мадагаскаре, где они эволюционировали как потребители экскрементов лемуров. Популяции лемуров сокращаются вследствие антропогенного влияния, что заставляет навозных жуков переходить на другие источники питания. Для оценки питания гигантского мадагаскарского навозника *Helictopleurus giganteus* (Harold) мы изучили ампликон-метагеномными молекулярными методами содержимое кишечника семи экземпляров этого вида из разных локалитетов. Мы обнаружили прочтения ДНК пяти видов млекопитающих, причем более 99% от общего числа прочтений принадлежат человеку и корове. В образцах не было обнаружено маркеров нативных Мадагаскарских млекопитающих. Полученные результаты

* Corresponding author / Автор-корреспондент

подтверждают антропогенное изменение рациона *H. giganteus*, хотя их следует интерпретировать с осторожностью, поскольку неизбежные контаминации могут в значительной степени способствовать высокому процентному содержанию прочтений ДНК коровы и человека.

Ключевые слова: метагеномика ампликонов, анализ содержимого кишечника, копрофагия, навозные жуки, Мадагаскар, секвенирование нового поколения, скарабеины

INTRODUCTION

Dung beetles (Coleoptera, Scarabaeidae, Scarabaeinae) are important elements in the food webs of ecosystems in Madagascar, where they originally evolved as consumers of lemur excrements (Orsini et al. 2007; Wirta and Montreuil 2008; Wirta et al. 2008; Viljanen et al. 2010; Wirta et al. 2010). The increased anthropogenic pressure reduces forest habitats, where the bulk of dung beetles live, and the population of lemurs, the original producers of food for the beetles. This causes dung beetles to shift to other food sources, which dramatically affects their distribution, population size, survival, and, as a rule, significantly rearranges tropical food chains (Hanski et al. 2008; Rahagalala et al. 2009; Wirta et al. 2014). In some cases, these changes in food webs can have a positive effect, leading to an expansion of the range and a change in the habitat. However, there is evidence that, in most cases, these effects are negative, as they lead to a reduction in habitats and the disappearance of populations of dung beetles and, consequently, to ecosystem degradation.

Assessment of the trophic association of dung beetles and their food producers until recently was done by indirect methods: field observations or collecting experiments using traps baited with dung of selected animals. The development of the high throughout sequencing (NGS) provides possibility to directly examine the diet of beetles by sequencing the DNA extracted from their gut. Short but specific DNA markers can be amplified by standard PCR protocol to limit DNA sequencing to mostly those taxa that are of interest for the researcher, for example, mammals (Kerley et al. 2018; Drinkwater et al. 2021). The amplicon metagenomic method was shown as a promising tool for the lemur inventories in Madagascar (Frolov et al. 2023). However, the trophic associations of different dung beetle species and their food producers are still poorly known. Apparently, many dung beetle species are generalists and can feed on dung of different mammals occurring in their range. Three *Helictopleurus* Orbigny, 1915, species, *H. marsyas* (Olivier, 1789), *H. neoamplicollis* Krell, 2000, and *H. quadripunctatus* (Olivier, 1789), have shifted to the cattle dung in open habitats (Hanski et al. 2008). The goal of the present paper is to report the results of amplicon metagenomic analysis of the gut content of seven specimens of *H. giganteus* (Harold, 1869), the largest and one of the most prominent dung beetles in Madagascar.

MATERIALS AND METHODS

Sampling localities, material, and collecting methods

Beetles were collected in four localities in central Madagascar (Table 1). The collecting localities are described in detail by Akhmetova et al. (2023). The beetles were collected from cow dung pads and by standard pitfall traps (Brown and Matthews 2016) baited with human feces. A trap was a 1-liter plastic container ca. 10 cm in diameter buried in the 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 of traps, they were covered with plastic lids attached 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. Voucher specimens are housed in the collection of Zoological Institute, Saint Petersburg, Russia (ZIN). About 30 collected beetles were dissected under a stereomicroscope and from 19 specimens with visible gut content the hindguts were dissected and placed in Eppendorf tubes with 96% ethanol.

DNA extraction and sequencing

DNA was extracted with FastDNA spin kit for soil (MP Biomedicals) according to manufacturer protocol. The extracted DNA was quantified using a Qubit fluorimeter 4.0 with high-sensitivity reagents

Table 1. Collecting details of the examined *Helictopleurus giganteus* specimens.

Sample no.	locality	biotope	coordinates	collecting method	date
1, 2, 3	Analamazaotra Special Reserve	primary forest	18°55'59"S 48°25'12"E	pitfall traps with human feces	17–20.II.2022
4, 5	Ankaratra massif, NW of Andraraly village	degraded primary forest	19°21'20"S 47°18'18"E	pitfall traps with human feces	23–26.02.2022
6	Foothills of Ankaratra massif, near Andraraly village	grassland	19°22'29"S 47°21'15"E	cow dung	23–24.II.2022
7	Mantadia National Park	primary forest	18°49'32"S 48°26'5"E	pitfall traps with human feces	18–20.II.2022

(Lumiprobe QuDye dsDNA HS Assay Kit) and 1 µl of DNA. Seven samples with highest concentration of the extracted DNA were selected for high throughput sequencing along with a control sample (distilled water). For amplicon metagenomic sequencing the following primer pair were 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. Library preparation and sequencing was done separately in two replicates. DNA extraction was performed at Chromas Core Facility, Saint Petersburg State University (Petergof, Russia), 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). Merged reads were length selected to filter off reads longer than 160 bp and shorter than 140 bp, to retain only sequences from target taxa. Primers were trimmed and the reads were quality filtered with -fastq_maxee 1.0 option. These procedures retained around 98% of raw reads for each sample. 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). Because both methods produced similar results, only ZOTUs were used for downstream analysis. ZOTUs of both replicates of each sample were compared and those ZOTUs occurred only in one replicate were discarded. ZOTUs were then manually annotated with BLAST (https://blast.ncbi.nlm.nih.gov) using megablast algorithm against nucleotide database.

RESULTS

Illumina sequencing yielded 899835 reads for seven samples in two replicates; control sample yielded no reads. After quality filtering and trimming, we obtained 897568 reads (over 98% of raw reads). Denoising with usearch revealed 42 ZOTUs. Discarding ZOTUs occurred only in one replicate reduced the total number of reads to 885129 (mean 63224) and number of ZOTUs to 21. After annotation of these ZOTUs with BLAST, one ZOTU returned no hits and 12 ZOTUs were annotated as human pseudogenes (numts). After these ZOTUs were also discarded, seven ZOTUs retained which comprised 866228 reads (mean 61873). They belonged to five mammal species. The reads were then grouped by species and the mean values of both replicates per sample were calculated as well as percentage (Table 2).

DISCUSSION

The present results are congruent with those obtained in the previous study (Frolov et al. 2023). All samples in the present analysis, regardless of the collecting locality and methods, yielded similar composition of amplicon reads, with almost all reads

Table 2. Results of amplicon metagenomic gut content analysis of *Helictopleurus giganteus*. For sample details see Table 1. Data are given as a number (#) and percent (%) of reads per sample.

Mammal species	Sample (beetle specimen)															
	1		2		3		4		5		6		7		mean	
	#	%	#	%	#	%	#	%	#	%	#	%	#	%	#	%
Bos taurus	57270.5	88.2	34371.5	56.3	27006.5	43.3	53243	85.8	29001.5	50.2	21847.5	35.3	20102.5	31.8	34691.9	55.9
Homo sapiens	7096.5	10.9	26450	43.3	34423	55.2	8277	13.3	28436	49.3	40107	64.7	42993	68.1	26826.1	43.6
Rattus norvegicus	529	0.8	0	0	885.5	1.4	0	0	23	0.0	0	0	34.5	0.1	210.3	0.3
Sus scrofa	0	0	185.5	0.3	0	0	510.5	0.8	169.5	0.3	0	0	0	0	123.6	0.2
Equus caballus	0	0	48	0	0	0	9.5	0.0	93.5	0.2	0	0	0	0	21.6	0.0
Total	64896	100	61055	100	62315	100	62040	100	57723.5	100	61954.5	100	63130	100	61873.4	100

belonging to human and cow. The proportion between the two is from 9/10 to 1/3 with mean values about 45% and 55%, respectively. The reads of three other mammal species comprise less than 1% in total, and are probably a result of contamination. No indigenous mammals, specifically lemurs, were recovered. Along with the fact that the beetles examined were collected from cow dung or attracted to the baited traps, this supports the idea that *H. giganteus* shifted its food preferences, which resulted from the colonization of the island by humans. However, our results should not be interpreted so that most or all food of *H. giganteus* comes from these two mammal species only, and that these species contributed equally to the beetle diet. Unavoidable contaminations may contribute reasonably to the high yield of the cow and human reads. The problem with contaminations may be further complicated by the possibility that human feces, utilized by beetles as food, included traces of human food, for example beef or pork; this may result in false positive indication of cow or swine dung as a food source for the beetles.

Drinkwater et al. (2021) experimented with laboratory feeding of a Bornean dung beetle species, *Catharsius renaudpauliani* Ochi et Kon, 1996. They examined the beetle gut content by real-time PCR and found that mammal DNA copy number declined with time post-feeding; after six hours post-feeding DNA copy number decreased drastically. The gut content of beetles collected in the field is highly unpredictable due to various factors. It is unknown when the beetle fed before capture. Our experience with dissecting a few dozen of beetles showed that visible gut content varied reasonably among individuals. Even though most specimens did have their hindguts filled with food, the success of the amplification may vary depending on the quality of the DNA fragments containing the target marker. Contaminants may be more readily amplified due, for example, to a lesser degradation.

Although the amplicon metagenomics method was shown as a promising tool for the lemur inventories in Madagascar, further research is needed to evaluate the possibility to use different Madagascan dung beetle species as "tools" for inventories of threatened native mammals. As the new food sources are more readily available than the lemur feces throughout the island (except probably for large protected areas with strict conservation regime), we think that many dung beetle species are not specifically attracted to lemur feces and feed on them occasionally in some biotopes. The poor knowledge of the dung beetle specialization on the dung of certain mammal species (or, as opposed, generalist feeding behavior) may negatively affect our attempts to use dung beetles as indicators of the presence of lemurs.

ACKNOWLEDGMENTS

We are thankful to the staff of Malagasy Institute of Conservation of Tropical Ecosystems (MICET) for the support in the acquisition of permits and logistic support during the expedition to Madagascar. Olivier Montreuil (National Museum of Natural History, Paris, France) confirmed identification of the beetle species. Sergey Tarasov, Michele Rossini, Fernando Lopes and Thomas Merrien (Finnish Museum of Natural History, Helsinki,

Finland) helped with fieldwork. Alexander Dyomin (Saint Petersburg State University) and two anonymous reviewers commented on the draft manuscript.

This research was funded by Russian Science Foundation, grant no. 22-24-00715. The insect collection of ZIN is maintained within the framework of the Russian State research project 122031100272-3.

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