Metabarcoding on an empty stomach: using stomach swabs to investigate the diet of the Asian musk shrew, *Suncus murinus*

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Abstract:
Suncus murinus, a widespread Soricidae, has been introduced to several islands, strongly impacting endemic species. Its dietary habits, especially at its introduction sites, are poorly understood and the level of impact on native taxa remains unknown. Obtaining dietary samples from shrews is challenging because of their high metabolic rates and rapid digestion. We tested the effectiveness of stomach swabs in analysing the diet of *S. murinus* using DNA metabarcoding on 300 individuals from Reunion Island (Western Indian Ocean). Non-target DNA amplification was substantial. We identified five preys belonging to three classes of Arthropoda (Arachnida, Insecta and Malacostraca) and one class of Annelida (Clitellata), with two of them assigned to species level, *Amynthas rodericensis* and *Pycnoscelus surinamensis*. Lycosidae and Malacostraca were the most frequent groups, each with a 50% frequency of occurrence. Stomach swabs provide insights into the dietary composition of *S. murinus*, but low DNA yield and purity limited detailed resolution. We highlight the importance of reducing the time lag between trapping and sample extraction and the use of blocking primers to prevent non-target amplification to enhance resolution of *S. murinus* diet composition.

Keywords: diet, alien species, next-generation sequencing, Soricidae, operational taxonomic units, Reunion Island.

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1 **Main Text**

2 Biological invasions are a major threat to biodiversity globally. Impacts of invasive alien species (IAS) on native
3 biota are manifold, including direct (i.e., competition for resources, predation) and indirect (i.e., ecosystem
4 functioning) impacts (Linders et al., 2019).

5 Dietary analyses are crucial for identifying predation pressure of IAS on native taxa (Egeter et al., 2019).
6 Traditional morphological approaches are limited by several factors such as prey type, prey size, level of digestion
7 and degree of mastication. Here, molecular methods, such as DNA metabarcoding, offer enhanced resolution and
8 the ability to detect soft, small and inconspicuous prey items, providing a more comprehensive dietary profile (Gil
9 et al., 2020).

10 The Asian musk shrew, *Suncus murinus*, is a small nocturnal mammal of the Soricidae family, native to south-
11 east Asia. Its close association to humans and efficient reproductive strategy have facilitated its rapid spread as an
12 IAS worldwide (Chang et al., 1999; Ruedi et al., 1996). Its arrival has been implicated in the loss of endemic
13 lizard species in Guam (Fritts and Rodda, 1998) and Mauritius (Solow et al., 2008).

14 Field observations and morphological diet analyses indicate that *S. murinus* is primarily insectivorous, but
15 opportunistically feeds on a variety of plants, invertebrates, and vertebrates (Varnham et al., 2002; Advani and
16 Rada, 1981). Detailed dietary investigations of shrews are impeded by their high metabolic rates which results in
17 rapid digestion of prey, yielding little material for stomach or faecal samples (Browett et al., 2023). While capture
18 by hand and immediate processing has resulted in good dietary information (Brown et al., 2014), this approach is
19 often impractical in challenging field conditions, where sampling immediately after capture is not always feasible.

20 Here, we use DNA metabarcoding from stomach swabs to investigate the diet of *S. murinus*. We conducted our
21 study on Reunion Island (Western Indian Ocean) where *S. murinus* was introduced in 1730 (Cheke, 1987) and
22 possibly threatens the critically endangered, micro-endemic Manapany day gecko, *Phelsuma inexpectata*. Despite
23 being present for almost 300 years, information about *S. murinus'* life history, diet and impact on island biota is
24 very limited. We performed stomach swabs on trapped shrews to investigate *S. murinus* diet and to assess possible
25 threat level of *S. murinus* on *P. inexpectata*. In doing so, we aim to provide a first insight into the dietary
26 composition of *S. murinus* on Reunion Island.

27 Diet samples were collected from *S. murinus* trapped in a 2-ha area in southern Reunion Island (21°06′ S, 55°36′
28 E). Trapping was done by the local NGO Nature Océan Indien, as part of an IAS control programme, using
29 unbaited INRA traps (BTT Mécanique, Roche-Lez-Beaupré, FR), strategically placed along rocks, tree roots and

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31 paths (Varnham et al., 2002) and left on site for the whole duration of the project (Nov 2019 - Nov 2021). Thirteen
32 capture sessions were held, each lasting between 4 – 15 days (9.5 ± 4.4, mean ± SD), including 127 traps with
33 daily checks (max. time trapped ~12h) resulting in 13,956 corrected trap nights and a capture rate of 2.44 ± 1.37,
34 mean ± SD, shrews per 100 trap nights. A total of 300 shrews were captured and euthanised using cervical
35 dislocation. Specimens were stored at -18 °C until dissection. Due to minimal stomach content, stomach walls
36 were swabbed to collect any possible remains of prey, preserved in 90% ethanol, and stored at -18 °C until DNA
37 extraction.

38 DNA was extracted using the E.Z.N.A. Tissue DNA Purification Kit (Omega Bio-Tek, Norcross, GA, USA),
39 following the manufacturer's guidelines. Due to low DNA yield, each extraction consisted in a pool of four swabs
40 from the same site, reducing the original 300 samples to 75 extraction samples. A short fragment (205 bp) of the
41 mitochondrial cytochrome c oxidase subunit I (COI) was amplified by PCR using the FwhF2-R2n primers from
42 Vamos et al. (2017), previously used to study the diets of bats (Mata et al., 2016), birds (da Silva et al., 2022) and
43 reptiles (Martins et al., 2022). Primers were modified to include Illumina adaptors and a 0–5 bp shift of Ns to
44 increase sequencing diversity and quality. PCR reaction comprised 5 µL QUIAGEN Multiplex PCR Master Mix
45 (Quiagen, Crawley, UK), 0.3 µL mix of each forward and reverse primers, 3.4 µL ultra-pure water, and 2.5 µL
46 DNA extract. Three PCR replicates were performed per sample. Cycling conditions consisted of an initial
47 denaturation step at 95 °C for 15 min, followed by 45 cycles of 95 °C for 30 s, 52 °C for 45 s, 72 °C for 20 s, and
48 a final extension at 60 °C for 5 min. All samples were successfully amplified when checked on 2% agarose gel.

49 Initial PCR clean-up was performed by a 1:3 dilution to remove primer dimer, followed by an indexing PCR using
50 2.8 µL ultra-pure water, 7 µL 2× Kapa HiFi, 0.7 µL each Index (P7/P5), and 2.8 µL cleaned PCR product. Cycling
51 conditions consisted of an initial denaturation at 95 °C for 3 min, 9 cycles of 95 °C for 30 s, 55 °C for 30 s, 72 °C
52 for 30 s, and a final extension of 72 °C for 5 min. A second bead clean-up using Agencourt AMPure XP beads
53 (Beckman Coulter, Brea, CA, USA) was performed to remove remaining primer dimer, nucleotides, and enzymes.
54 All purified PCR products were quantified using Epoch, followed by normalisation to 20 nM. The library was
55 quantified using qPCR (KAPA Library Quant Kit qPCR Mix; Bio-Rad iCycler), diluted to 4 nM and pooled
56 equimolarly for sequencing using a 300 cycles MiSeq Micro Kit (Illumina) for an average of 25,000 paired-end
57 reads per sample-marker combination. DNA extraction, library preparation and sequencing were performed in-
58 house at CIBIO, University of Porto, Portugal.

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60 Paired-end reads were aligned using PEAR (Zhang et al., 2014), rejecting base pairs with q-scores lower than 26
61 (Martins et al., 2022). Reads were assigned to samples, and primer sequences were removed using the command
62 *ngsfilter* in OBITools (Boyer et al., 2016), allowing four mismatches. Reads were de-replicated into unique
63 sequences or exact sequence variants (ESVs) and singletons were removed, using *obiuniq*. ESVs differing from
64 the expected 202–208 bp were excluded using *obigrep* and were denoised using *obiclean* with an 'r' level of one
65 to remove potentially spurious sequences. An Operational Taxonomic Unit (OTU) table was produced using
66 *obiannotate*, and a match-list with all the internal matches of OTUs was built using *usearch_global* from
67 VSEARCH (Rognes et al., 2016). Further cleaning using the R package LULU (Frøslev et al., 2017) removed
68 potential mtDNA nuclear copies and persisting errors. ESVs with a read count < 1% of the total number of reads
69 of each PCR product were discarded (Mata et al., 2016) and all reads identified in the extraction and PCR controls
70 were subtracted from the corresponding sample batch (Evans et al., 2021).

71 Taxonomic assignment of OTUs was done using both the Barcode of Life Database (BOLD) and the Basic Local
72 Alignment Search Tool (BLAST), with sequences below 90% similarity assigned to class, 90–95% to family, and
73 above 95% to species or genus level. BOLD is a reference database that provides taxonomic information and
74 allows comparisons of specimens to closely related species, while BLAST identifies species by comparing
75 nucleotide sequences against a database to find the closest matches. Non-animal taxa, internal parasites (phylum
76 Nematoda) and shrew sequences were removed from the OTU list.

77 The libraries generated ca. 7.7 million raw sequence reads. Non-target amplification was observed in both samples
78 and controls, with Nematoda accounting for 0.13% and *S. murinus* for 99.80% of total reads, respectively. This is
79 a common issue in metabarcoding (McInnes et al., 2017), especially when applied to swabs. After negative
80 controls, singletons, replicates, and taxa filtering, the final diet dataset consisted of 275,647 prey reads present in
81 26 out of 75 pooled extraction samples, belonging to three classes of Arthropoda and one class of Annelida, with
82 two OTUs assigned to species level, *Amynthas rodericensis*, an introduced earthworm, and *Pycnoscelus*
83 *surinamensis*, an introduced cockroach. Lycosidae and Malacostraca were the most frequent OTUs identified in
84 our extraction samples, each with a 50% frequency of occurrence, while the remaining OTUs occurred in 16,67%
85 of the samples (Table 1).

86 Annelids were reported from other Soricidae diet, with some species being highly specialised on earthworms
87 (Díaz de Pascual et al., 2000). *Suncus murinus* in the Indian desert has a strongly plant-based diet (Advani and
88 Rana, 1981), while in Pakistan it has a primarily insect-based diet (Roberts, 1977). In a similar study on *S. murinus*
89 from Mauritius, Brown et al. (2014) was able to retrieve 76 invertebrate prey OTUs, from captured and

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91 immediately processed shrews, belonging to three classes of Arthropods (Arachnida, Insecta and Malacostraca) 92 and one class of Mollusca (Gastropoda).

93 We did not detect vertebrate prey in the diet of *S. murinus*, including the Critically Endangered *P. inexpectata*. 94 However, the absence of vertebrate prey in our results does not confirm that *S. murinus* does not prey on 95 vertebrates, as our study had significant limitations. The low DNA yield from stomach swabs, host DNA 96 amplification bias, and the need for pooled samples likely reduced our ability to detect less abundant prey items. 97 Although identified prey species have mainly terrestrial habits, and *P. inexpectata* is mostly arboreal (Choeur et 98 al., 2023), predation cannot be ruled out.

99 *Suncus murinus* has been introduced to multiple islands across the Indo-Pacific, where it disrupts local biodiversity 100 through predation and competition (Solow et al., 2008; Fritts and Rodda, 1998). While metabarcoding is effective 101 for studying diet, constraints remain in the collection of samples from *S. murinus* that critically affect the results. 102 Our low number of OTUs is likely the result of empty shrew stomachs (Browett et al., 2023). We highlight the 103 importance of reducing the time lag between the capture and processing of trapped shrews to increase stomach 104 contents. While we used shrews from an IAS control programme, targeted sampling with minimal time lag could 105 strongly improve dietary resolution while significantly reduce the number of samples needed (see Brown et al., 106 2014). Alternative trapping methods that facilitate the collection of uncontaminated faeces when logistical 107 constraints do not allow immediate processing should be investigated. Stomach swabs resulted in low DNA yield 108 and purity, necessitating pooled samples for extraction, and exacerbated host amplification and reduced prey data, 109 therefore, we recommend the use of optimized primers (Browett et al., 2023) and blocking primers to prevent 110 non-target amplification and enhance data resolution and accuracy.

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115 **Competing interests**

116 The authors have no competing interests to declare.

118 **Author contributions**

119 Conceptualization: MAR, SD, CR; Methodology: MAR, ArC, MS, SD, CR; Data collection: MAR, AiC, CB,
120 ArC, AG, NH, MS; Formal analysis and investigation: MAR, AiC, CR; Writing - original draft preparation: MAR,
121 CR; Writing - review and editing: MAR, AiC, CB, ArC, AG, NH, MS, SD, CR; Funding acquisition: MAR, CB,
122 MS, SD.

123 **Ethics declarations**

124 Samples used in this study were collected from shrews that were trapped during an invasive alien species control
125 programme led by the local NGO Nature Océan Indien, with methods approved by the *Direction de*
126 *l'environnement, de l'aménagement et du logement* de La Réunion and in accordance with the French law, *Code*
127 *de l'environnement* R411-46 & R411-47 and *Code rural et de la pêche maritime* R214-98.

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Tables

Table 1. Identified prey OTUs in the diet of *Suncus murinus* based on DNA metabarcoding from stomach swabs. Prey was detected in 26 of 75 pooled extraction samples. Detected OTUs with taxonomic classification and the frequency of occurrence (%) of each prey item consumed are given.

Phylum	Class	Order	Family	OTU	Frequency of Occurrence
Annelida	Clitellata	Crassiclitellata	Megascolecidae	*Amynthas rodericensis*	16,67%
Arthropoda	Arachnida	Araneae	Lycosidae	Lycosidae	50,00%
Arthropoda	Insecta	Blattodea	NA	Blattodea	16,67%
Arthropoda	Insecta	Blattodea	Blaberidae	*Pycnoscelus surinamensis*	16,67%
Arthropoda	Malacostraca	Isopoda	NA	Isopoda	50,00%

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Tables

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Identified prey OTUs in the diet of Suncus murinus based on DNA metabarcoding from stomach swabs. Prey was detected in 26 of 75 pooled extraction samples. Detected OTUs with taxonomic classification and the frequency of occurrence (%) of each prey item consumed are given.

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