Metabarcoding gastrointestinal nematodes in sympatric endemic and non-endemic species in Ranomafana National Park, Madagascar

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Abstract

While sympatric species are known to host the same parasites species, surveys contrasting parasite assemblages between sympatric species are rare. To understand how parasite assemblages between sympatric host species differ in a given locality, we used a non-invasive identification method based on high-throughput sequencing. We collected fecal samples from mouse lemurs and sympatric species in Ranomafana National Park, Madagascar, during 2010-2012 and identified their parasites by metabarcoding; sequencing the small ribosomal subunit (18S) gene. Our survey included 11 host species, including: endemic primates, rodents, frogs, gastropods and non-endemic black rats and dogs. We identified nine putative species of parasites between host species, although their correspondence to actual parasite species is not clear as the resolution of the marker gene differs between nematode clades. For the host species that were successfully sampled with ten or more positive occurrences of nematodes, i.e., mouse lemurs, black rats and frogs, the parasite assemblanges differed significantly between host species, sampling sites and sampling years. Our metabarcoding method shows promise in interrogating parasite assemblages in sympatric host species and emphasizes the importance of choosing marker regions for parasite identification accuracy.

Keywords: Lemurs, Metabarcoding, Parasites, Invasive species, Non-invasive sampling

Running title: Metabarcoding parasite assemblages in sympatric host species

Introduction

Parasite dynamics research is hindered by parasite groups that are difficult to identify; requiring extensive taxonomical expertise. Furthermore, the identification of intestinal nematode species traditionally requires dissection of host animals to collect and morphologically identify adult nematode specimens. This approach is time-consuming and due to its' invasiveness, is not always feasible.

The standard method for assessing gastrointestinal parasites non-invasively is fecal analysis (Gillespie 2006). When identification is based on egg or larval morphology, this often leads to parasite identification at high taxonomical levels, such as order or family, and rarely allows for identification at the genera or species-level. Several procedures based on molecular markers have been proposed for non-invasive assessment of parasitic nematodes (e.g.: Wimmer et al. 2004). Although these can reliably identify specific species or strains, the published procedures lack the broad spectrum needed for host populations of unknown parasite communities. Barcoding, i.e., identifying species by sequencing a marker gene, is the method of choice to identify high diversity among nematode communities. Furthermore, high-throughput sequencing allows for the identification of several nematode taxons from a single fecal sample, i.e., metabarcoding (Aivelo and Medlar 2017; Taberlet et al. 2012), but few studies have used this method to identify gastrointestinal nematodes (Avramenko et al. 2015; Lott et al. 2015; Tanaka et al. 2014). As species are not defined by sequence, the groupings resulting from barcoding analyses are referred as operational taxonomic units (OTUs) (Blaxter et al. 2005). OTUs may not correspond to actual species but to taxons of lower or higher level (Bik et al. 2012).

While parasite communities in sympatric primates have already been studied (e.g., Kouassi et al. 2015; Loudon and Sauther 2013; Maldonado-López et al. 2014; Muriuki et al. 1998; Petrášová et al. 2010; Pourrut et al. 2011; Schwensow et al. 2010; Teichroeb et al. 2009; Trejo-Macías et al. 2007; Trejo-Macías and Estrada 2012), there have been relatively few studies comparing primate parasite species composition to sympatric non-primate mammals. Nevertheless, parasite sharing appears to be common in wild mammals (Chakraborty et al. 2015; Dallas and Presley 2014; Kouassi et al. 2015). Parasite communities can also be affected by the introduction of non-endemic host species that provide new competent hosts for endemic parasites (Dunn *et al.* 2012; Kelly *et al*. 2009) or they can bring new parasite species to the ecosystem (Hudson and Greenman 1998; Taraschewski 2006). Introduced hosts tend to have lower parasite species diversity than in their endemic area (Dobson and May 1986; Freeland 1983; Torchin *et al*. 2003), which may be due to loss of their original parasites during colonization (MacLeod et al. 2010).

To our knowledge, there have been no studies on metabarcoding intestinal parasites from different sympatric host species. Our principal aim was to assess whether metabarcoding is a viable tool for such parasitological surveys. We explored gastrointestinal nematode assemblages in several species living within or in the peripheral zone of Ranomafana National Park, Madagascar. Using invasive black rats, we also tested if molecular identifications from larvae acquired from fecal samples matched morphological identifications made from adult nematodes. Ranomafana National Park is a suitable ecosystem for study, as it has high biodiversity, including 13 primate species, with notable anthropogenic disturbance and contains several non-endemic mammalian species. In Ranomafana National Park, a number of endemic species are threatened with extinction, including critically endangered golden and

greater bamboo lemurs (*Hapalemur aureus* (Andriaholinirina et al. 2014a) and *Prolemur simus* (Andriaholinirina et al. 2014b), respectively). We hope that our method could help conservation efforts and facilitate wildlife health assessment within biodiversity hotspots. The research questions were: i) how well the 18S marker gene can be used to survey intestinal parasite assemblages and ii) do non-endemic and endemic host species have similar nematode assemblages. We expected similar parasite assemblages between closely related species and between species sharing the same ecological niches, i.e., terrestrial species would have more overlap with each other compared to arboreal species.

Methods

Sampling

We collected fecal samples (Table 1) from sympatric species from September to December in 2010, 2011 and 2012 in southeastern Madagascar (21°16' S latitude and 47° 20' E longitude). The national park is established on lowland to montane rainforest between 500 and 1500 meters elevation. The park consists of 43500 hectares of protected area as well as a peripheral zone with limited protection (Wright and Andriamihaja 2002). We collected mouse lemur samples nightly from two different transects, the first one within the National Park and the second on the periphery of the park in Centre Valbio's campsite. We laid 50 live traps (22.2 x 6.6 x 6.6 cm; XLK, Sherman Traps Inc., Florida USA) along a trail at 50 meter intervals, an hour before sunset. Black rats (*Rattus rattus*), snails (Gastropoda sp.) and endemic rodents (*Nesomys audeberti* and *Eliurus* spp.) were also caught as a side catch in the same traps. We additionally used these two transects for opportunistic sampling of medium-sized lemurs

(*Eulemur rubriventer, Hapalemur aureus, Prolemur simus*), domesticated dogs which range freely within the local village and forested areas (*Canis lupus*) and frogs (*Ptychadena* spp. and *Mantidactylus* spp.). We collected black rat samples from an additional location on the peripheral zone of the park near Ambatovory. All sites contained secondary forest growth with endemic and non-endemic trees.

We collected the traps three hours after sunset, sampled feces from the traps and brought any captured black rats and mouse lemurs to the laboratory of Centre Valbio. We washed the traps after each use and dried them in sunlight to decrease the chance of contamination from previous captures. We terminated invasive black rat specimens and examined a subset (n=17) for adult nematodes in the gastrointestinal tract. We dissected the rats, opened their gastrointestinal tract from stomach to anus, observed the gut lining and contents under a microscope in saline solution and collected all helminths.

Ethical note

We minimized the duration that animals were kept in captivity, especially during the mouse lemur mating season. We released the mouse lemurs as soon as we had collected the data from the individual. We identified and released other captures on-site. We handled mouse lemurs under red light to minimize stress.The procedures used were consistent with ethical standards and approved by the trilateral commission (CAFF/CORE) in Madagascar (permits: 203/11/MEF/SG/DGF/DCB.SAP/SCBSE and 203/12/MEF/SG/DGF/DCB.SAP/SCBSE)

DNA isolation and sequencing

After collecting the fecal matter, we used Baermann's method to isolate the nematodes

(Baermann 1917). We placed the fecal matter on a tissue (one half of 1-ply Kimwipe, Kimberly-Clark Europe Ltd., Surrey, United Kingdom), folded the tissue and tied it with string. We then placed this packet on a sterile glass funnel which was filled with approximately 37°C distilled water. This allows all the living nematode larvae to swim out of the fecal matter into the water. We collected the samples two days later, centrifuged them for 5 minutes at 2800 rcf and discarded the supernatant. We quantified the number of nematode larvae by examining the pellet under the microscope and stored the larvae in 70% ethanol in a freezer at -18°C. It should be noted that Baermann's method only isolates nematodes which have a free-living stage and thus we, therefore, could not acquire entire nematode communities. We refer to the partially resolved parasite communities as assemblages. We tested approximately every fifth rat fecal sample (n = 18) after Baermann extraction by visual screening on flotation liquid and did not find any residual nematode parasites.

For nematode DNA extraction, we used half of the visible larvae mass; approximately 40 microliters of liquid. For DNA extraction, we centrifuged the sample and removed any ethanol. For adult nematodes collected directly from dissected rat intestine, we used one individual or a part of an individual. The sample was incubated for 2 hours at room temperature in milliQ water to rehydrate the nematodes and remove excess ethanol. To lyse the cells, we centrifuged the sample, removed the water and incubated the sample in 400 microliters of TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.5) together with 40 micrograms of proteinase K overnight at 56ºC. We collected DNA with isopropanol precipitation: we centrifuged the samples for 10 minutes at 15000 rcf and discarded the pellet. We mixed the supernatant with 400 microliters of isopropanol and incubated for 5 minutes at room temperature. After precipitation we centrifuged the samples for 10 minutes at 15000 rcf,

discarded the supernatant and washed the resultant pellet twice using 500 microliters of 70% (v/v) ethanol. After ensuring that all ethanol had evaporated, we suspended the sample in 30 microliters of TE buffer.

To amplify the ribosomal small subunit gene (18S) we used primers from Bhadury and Austen (2010): M18F: 5′-AGRGGTGAAATYCGTGGAC-3′ and M18R: 5′-TCTCGCTCGTTATCGGAAT-3′. These primers were designed for marine nematodes with high-specificity and minimal co-interference from other eukaryotes. The PCR mix included 1 unit Phusion high-fidelity polymerase (Thermo Fisher Scientific, Inc, Waltham, MA, USA) with buffer, 10-100 ng (0.5-5.0 microliter) of template, 0.2 µM primers, 200 µM dNTP mix, 1.5 mM MgCl2 and 2% DMSO per reaction. The PCR program included initial denaturation at 98^{o}C for two minutes, then 30-40 cycles of 15s denaturation at 98^{o}C, annealing at 53^{o}C for 30s and 30s extension at 72^{o}C and ending with 10 minutes of final elongation at 72^{o}C. PCR results were checked on a 1% agarose gel.

Amplicons were sequenced at the DNA sequencing and Genomic laboratory, Institute of Biotechnology, University of Helsinki using a Roche 454 Genome Sequencer FLX+.

Sequence analysis

We performed data analysis using the Séance pipeline for reference-based phylogenetic amplicon analysis (Medlar *et al*. 2014). We used Ampliconnoise (ver. 1.29) (Quince *et al.* 2011) to denoise each sample. We discarded sequences with ambiguous base calls, more than 1 error in the multiplexing barcode or more than two errors in the primer sequence, removed multiplexing barcodes and primers and truncated all sequences to 250bp. We removed

putative chimeric sequences using UCHIME (ver. 4.2.40) in *de novo* mode (Edgar *et al.* 2011) and excluded all sequences with a copy number less than 5. We expect that a majority of the sequences filtered out will represent PCR artefacts and sequencing errors not caught during preprocessing. We performed the clustering of the sequences with a similarity threshold of 99%. Séance's clustering methodology explicitly models homopolymer length uncertainty in 454 data across many samples.

Clusters were labelled using Séance's taxonomical labelling strategy. In brief, each cluster is formed around a (generally highly abundant) centroid sequence, which we use to perform a MegaBLAST (Camacho et al. 2009) search of the NR (non-redundant) database at NCBI. We excluded results with lower than 90% identity and those from environmental and metagenomic samples. As each sequence is only 250bp long there is often some ambiguity as to which species it is most similar to, so instead we report the lowest common ancestor from the NCBI taxonomy of all top scoring BLAST hits (i.e., the taxon which contains all the taxons representing the top hits). For comparison, we generated labels using the same procedure, but substituting the NR database with SILVA (SSURef NR ver. 115) (Quast *et al.* 2013), which contains its own taxonomic data.

Séance uses a phylogenetic placement strategy for phylogenetic analysis. For this we need a reference tree to extend with the cluster sequences. To build the reference tree, we extracted the complete 18S rRNA gene sequence from all 1320 members of the phylum Nematoda found in SILVA ver. 115 and built a tree with RAxML (ver. 7.2.8) (Stamatakis 2006). RAxML was run with the GTR+gamma substitution model for 10 repetitions. We used Séance's phylogenetic placement command to place the cluster centroid sequences into the reference

tree. Visualizations were also produced with Séance.

Putative species determination

As the resulting OTUs may include non-nematode, contamination, and spurious OTUs caused by amplification or sequencing errors, we extracted what we termed putative species from the results of each cluster analysis. To generate a putative list of nematode species, we first removed all clusters with taxonomic labels to phyla other than Nematoda. To ensure that we report only nematodes parasitic to the host species we sampled, we studied OTU co-occurrence patterns, e.g., known dipteran parasitizing nematode clusters were removed as they were only found in samples together with dipteran clusters. Free-living nematodes may have contaminated our samples, for example, by attaching to a rodents' foot and then transferring to the feces prior to collection. OTUs were deemed to be contamination from soil nematodes when the best hits for clusters were soil nematode groups and there was a reasonable chance of contamination. Finally, it is likely that there is a number of spurious OTUs due to amplification and sequencing errors. To conservatively take these into account, we merged OTUs that formed a homogenous group. The criteria for merging was that a) the OTUs were clustered to the same taxon, b) they formed a monophyletic group in the phylogenetic tree, c) there was one clearly dominant OTU in this group and d) the OTUs occurred in the same individuals (so-called head-tail structure (Porazinska, Giblin-Davis, Sung, et al. 2010; Figure S1). Nevertheless, it should be noted that these putative species can contain more than one parasite species or, theoretically, they can also reveal cryptic species of parasites, i.e., one parasite species can be divided into two or more putative species.

Data availability

The raw sequences have been deposited in the Sequence Read Archive under SRA number SRP042187. The metadata for the samples, including the matching of samples to sample accession numbers can be found in the data file in Figshare: doi: 10.6084/m9.figshare.1289310

Statistical analysis

We performed all statistical tests and their visualizations in R using the stats package (R Core Team 2013) and the mvabund package (Wang et al. 2016).

To assess the resolving ability of the particular primers we used, we extracted all nematode 18S sequences from the SILVA database, extracted the marker region using the primers, trimmed the sequences to 250bp and clustered them at 99% similarity. Then we assigned labels for each of the clusters and quantified the number of unique clusters (i.e., clusters composed of different sets of sequences) within each taxon corresponding to our observed putative species labels.

We calculated parasite prevalence for host taxa with 10 or more samples and analyzed parasite assemblages in host taxa with 10 or more successful sequencings (mouse lemurs, black rats and frogs) by using a generalized linear model with a binomial link function and using trapping site and year as variables in addition to host species. As we were not able to identify putative species in all positive samples (i.e., samples without successful sequencing), we removed a similar proportion of negative samples from the analysis. P-values are assigned by resampling which bootstraps probability integral transform residuals.

Results

We collected a total of 872 samples, of which 571 contained nematodes and 249 were successfully sequenced (Table 1). We dissected 17 black rats of which 14 were positive for nematodes in the gastrointestinal tract. The fecal samples of these 14 rats were also positive for nematodes. The remaining three rats were all correspondingly negative based on their fecal samples. There were two distinct morphotypes of nematodes: in the first two thirds of the small intestine we collected nematodes resembling *Nippostrongylus* sp. (n=14) and from the stomach, nematodes that resembled *Mastophorus* sp. (n=2).

Sequencing and sequence analysis

The amplification and sequencing success rates were variable, ranging from 100% success in gastropods to 0% in *Eliurus* and *Nesomys* spp. (Table 1). If amplification did not succeed on the first try, we attempted reamplification. If amplification was still unsuccessful, we reisolated the DNA and amplified it again. For the larval samples of mouse lemurs, there was approximately 30% success in the first isolation and 22% success on the second isolation.

Table 1: Number of collected samples from study species in Ranomafana National Park, Madagascar between September 2010 and December 2012, and species information regarding if species are arboreal (A) or terrestrial (T), nocturnal (N), cathemeral (C) or diurnal (D), omnivores (O) or herbivores (H) (Nowak 1999a, 1999b) and their sample counts and sequencing successes.

		Niche	Activity	Feeding	Total number of the samples	Positive for nematodes	Successful sequencing	Nematode prevalence (%)
Rufous mouse lemur	*Microcebus rufus*	A	N	O	632	469	212	74
Red-bellied brown	*Eulemur*	A	C	H	7	3	1	

lemur	*rubriventer*							
Golden bamboo lemur	*Hapalemur aureus*	A	D	H	4	3	1	
Greater bamboo lemur	*Prolemur simus*	A	D	H	9	1	1	
Tufted-tailed rats	*Eliurus spp.*	A,T	N	O	82	3	0	4
	Nesomys spp.	T	D	H	21	2	0	10
Black rat	*Rattus rattus*	T,A	N	O	68	37	18	54
	- *dissections*				17	14	5	
Dog	*Canis lupus*	T	C	O	5	4	2	
Frogs	Ranoidea	T	N	O	40	20	12	50
Snails	Gastropoda sp.	T	C	H	4	2	2	

We had a total of 677,451 reads from 290 samples. After preprocessing we had 409,088 high quality reads, which were comprised of 7,308 unique sequences. The median number of high quality reads per sample was 722 with an inter-quartile range of 279-2098. When all sequences with copy number less than 5 were removed, we had a total of 308 unique sequences, which is representative of 97.3% of the reads that passed quality control. We performed sequence clustering with a similarity threshold of 99%, which resulted in 35 OTUs. Of these OTUs 16 had a taxonomic label other than Nematoda. Most of the contamination was most likely due to dipterans laying eggs in the samples during processing. One OTU co-occurred only with dipteran contamination and was labelled as *Howardula* sp., a nematode species parasitic in flies. This OTU was therefore classified as contamination. Furthermore, there were 3 OTUs labelled as soil nematodes and recovered only with contact to the soil. There were also matches to the soil nematodes in samples directly collected from

rodents without contact to the soil and these were deemed to be parasitic nematodes (2 OTUs).

After processing, we had 9 putative species (Table 2; Figure 1).

Resolution and reliability of putative nematode species

The putative nematode species were named using the lowest common ancestor in the NCBI taxonomy for all top scoring BLAST hits for the centroid sequence. With the exception of PS3 and PS4, the putative nematode species were labelled to the genus level. However, some of these matches were free-living nematode genera, like PS2 (*Caenorhabditis*) and PS6 (*Panagrellus*) (Table 2). We performed labelling also with a curated database (SILVA) and the results were concordant, but more conservative than with NCBI NR (Table 2). After quality control and curation, we had a total of 254 samples which included parasitic nematodes (Table 1). The resolving ability of the primers differs substantially between putative species labels (Table 2): e.g., the clusters labelled Chromadorea could consist of 2 to 103 species, while two *Rhabditoides* spp. (which belong to Chromadorea) clusters have only one described species in them.

To assess the reliability of using Baermann's method, i.e., larvae developed from the fecal samples, as a proxy for which adult specimens are present in the gastrointestinal tract, we compared the putative species from the dissected host black rat individuals in which we got successful sequencing from both larval and intestinal samples. *Nippostrongylus*-like adult specimens and the majority of the corresponding larval amplicons belonged to PS3 (Strongylida): two of the larval samples corresponded to their respective adult intestinal

nematodes, but one larval sample did not contain the expected PS3 but rather PS1 (*Strongyloides*). The nematodes identified as *Mastophorus* sp. did not occur in larval samples though it amplified well from the two adult gastrointestinal samples.

Table 2: Putative species and their potential taxonomic labels from study host species in Ranomafana National Park, Madagascar between September 2010 and December 2012. There is a wide difference between the taxonomic resolution of the lowest common ancestor of the top scoring BLAST hits in NR and SILVA database. Some species are resolved to genera level (like *Strongyloides* and *Syphacia*), while other samples are resolved to much higher taxa (like Chromadorea and Strongylida). The SILVA database gives more conservative labels. The next two columns include the closest BLAST match for the centroid sequence and other close BLAST matches. The rightmost column gives the number of unique clusters resulting from clustering all samples contained in SILVA database in a lowest common ancestor taxon and total number of sequences with the taxon.

Putative species	LCA from NR database	LCA from SILVA database	Centroid BLAST match	Other close BLAST matches	Unique clusters / sequences in SILVA
1	*Strongyloides*	*Strongyloides*	*S. stercoralis*	*S. procyonis*	3/10
2	*Caenorhabditis*	*Caenorhabditis*	*C. elegans*	several *Caenorhabditis* spp.	3/3
3	Strongylida	Rhabditidae	*Gurltia paralysans* / *Dictyocaulus*	*Strongylus, Filaroides, Trichostrongylus, Ancylostoma, Angiostrongylus*	53/135
4	Chromadorea	Chromadorea	*Physaloptera thalacomys*	*Gongylonema pulchrum*	147/291
5	*Enterobius*	*Enterobius*	E. vermicularis		1/1
6	*Panagrellus*	*Panagrellus*	*P. redividus*		4/8
7	Rhabditoides	*Rhabditoides*	*R. regina*		2/2
8	*Raillietnema*	Chromadorea	*Raillietnema* sp.	*Cosmocercoides*	147/291
9	*Phasmarhabditis*	Rhabditidae	*Phasmarhabditis* sp.		53/135

Patterns of putative species distribution

For most of the host species, the sample numbers were quite low and we therefore did not manage to sample all of the putative species in these hosts. PS3 was the only putative species in three larger sized lemurs, brown and bamboo lemurs, whereas dogs also had PS6 (Figure 1). Gastropods were the only host to contain PS9.

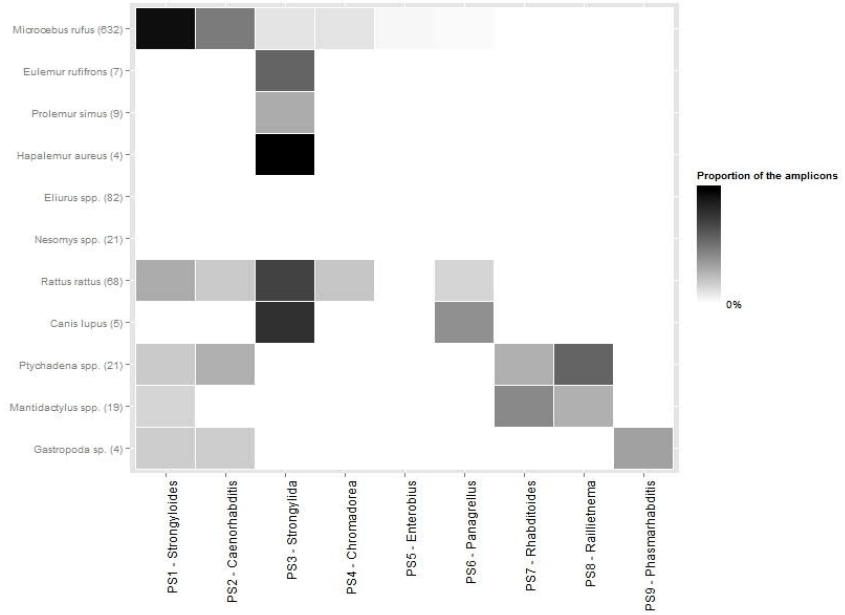

Figure 1: A heatmap with one host species per row and one putative species per column in Ranomafana National Park, Madagascar between September 2010 and December 2012. Numbers after the scientific name represents the sample size for each species. The scientific name after putative parasite species number represents the lowest common ancestor of top BLAST hits from SILVA database.

Of the taxa we sampled more than ten times, mouse lemurs, rats and frogs had nematode parasites in half or more of the fecal samples (Table 1). In contrast, despite high sample numbers, we found few parasites in endemic rodent species (*Eliurus* and *Nesomys)*, and we

were not successful in sequencing their parasites.

We used taxons with more than ten successful sequencings (mouse lemurs, black rats, frogs) to explore differences between parasite assemblages. All three variables, host species (LRT= 84.54, $p_{df=2} < 0.001$), sampling site (LRT = 27.23, $p_{df=2} = 0.008$) and sampling year (LRT= 75.27, $p_{df=1} < 0.001$) had a significant effect on parasite assemblage structure. In univariate tests between putative species and variables (Table 3), the differences between hosts were driven by PS1 and PS2, which were less prevalent in frogs (15% and 15%, respectively) and in rats (28% and 20%) than in mouse lemurs (79% and 61%), and PS7 and PS8, which are not present in rats or mouse lemurs, but have prevalence of 32% and 42%, respectively, in frogs. Furthermore, Fragment site differs in several putative species (PS1-4 and PS6), whilst Talatakely and Campsite sites do not significantly differ from each other for any putative species. The presence of PS1 and PS2 differs significantly between years.

Table 3: The statistical significance of each multivariate term specified in the fitted model using mvabund package from study species in Ranomafana National Park, Madagascar between September 2010 and December 2012. The test statistic is calculated with the Wald test and p-values are calculated with the PIT-trap method. The statistically significant ($p < 0.05$) values are marked with bold.

	Intercept		Host - *Microcebus*		Host - *Frogs*		Site - Fragment		Site - Talatakely		Year	
	Wald	p	Wald	p	Wald	p	Wald	p	Wald	p	Wald	p
PS1	**3.29**	**<0.001**	**3.631**	**0.004**	1.353	0.12	**0.038**	**0.01**	0.802	0.93	**3.282**	**0.007**
PS2	**6.04**	**<0.001**	1.170	0.38	1.52	0.12	**0.037**	**0.01**	0.454	0.93	**6.037**	**<0.001**
PS3	0.70	0.59	**5.563**	**<0.001**	0.077	0.12	**2.518**	**0.007**	1.557	0.48	0.699	0.59
PS4	**2.85**	**0.012**	0.076	0.60	0.069	0.12	**2.069**	**0.01**	0.161	0.93	**2.848**	**0.01**

PS5	1.43	0.29	0.064	0.60	0.002	0.34	0	0.66	0.737	0.93	1.433	0.29
PS6	0.86	0.59	1.157	0.37	0.061	0.12	**1.899**	**0.01**	0.325	0.93	0.865	0.59
PS7	0.00	0.59	**0.000**	**0.61**	0.073	0.12	0	0.66	0	0.93	0	0.59
PS8	0.00	0.59	**0.00**	**0.61**	0.075	0.34	0	0.66	0	0.91	0	0.59

Discussion

Our results show that metabarcoding can be used to non-invasively resolve the diversity in previously uninvestigated partial parasite assemblages. Non-invasive sampling and metabarcoding revealed differing parasite assemblages in sympatric species inhabiting the Malagasy rainforest. Nevertheless, the detection of the parasite sharing between different species was limited by the trade-offs inherent in the choice of the marker gene and sampling method.

We found statistically significant differences in parasite occurrence between host species, between years and between sampling localities (Table 3). While campsite and Talatakely were highly similar in parasite occurrence, more distantly situated forest fragments differed in the occurrence of PS1, PS2 and PS4. Parasite assemblage in black rats did not differ from frogs, but they did have significant difference to mouse lemurs (Table 3; Figure 1) This is mostly driven by a difference of degree, not difference of kind, as the host species have similar putative species. The mouse lemurs and black rats, the two most extensively sampled host species, seem to host almost identical groups of putative species, with the exception of PS5 (matched to *Enterobius*) which appeared exclusively, though rarely, in mouse lemurs (Figure 1). Nevertheless, as the resolution of the marker gene is limited, we do not know whether putative species contain one or more parasite species. That is, we do not know whether mouse lemurs and rats share parasite species or if the number of putative species is representative of

their actual parasite richness. In contrast, frogs were differentiated by the presence of frog-specific putative species. As the lowest common ancestor would suggest, these putative species belong to taxa previously known to infect amphibians and gastropods. While we sampled the endemic rodents well (*Eliurus* spp. and *Nesomys* spp.), we rarely detected parasites in the feces (Table 1), which means they probably have parasite species not detected by our method. While we are unable to identify all black rat parasites, their parasite communities differ, at least partially, compared to the endemic rodents. This result is in line with previous studies on the ectoparasites of endemic rodents and black rats, which showed that endemic rodents did not have any invasive fleas while they were abundant on black rats, especially on disturbed sites (Laakkonen et al. 2003).

In assessing the usefulness of parasite identification methods, whether it is a new metabarcoding method or traditional coproscopy, there are three distinct questions: i) how well methods detect parasite species, ii) how they resolve the number of parasitic taxa and iii) how accurate is the identification of these species. Mitochondrial cytochrome oxidase subunit I (COI) gene is the standard marker gene for barcoding metazoan species (Hebert *et al.* 2003), but it has proved impractical for nematodes. We used the ribosomal small subunit gene (18S) as the barcode for nematodes for several reasons: 18S has conserved primer sites across all nematodes, amplicons can be used for identification (Porazinska *et al.* 2009, Tanaka *et al.*, 2014) and it is the most sequenced gene region in nematodes. As this gene region is relatively conserved it underestimates species richness (De Ley *et al.* 2005; Tang *et al.* 2012). Nevertheless, for mouse lemur putative species richness, we are comparable with with previous studies (Raharivololona and Ganzhorn 2010; Raharivololona and Ganzhorn 2009) and our previous study suggests we sampled mouse lemurs exhaustively (Aivelo et al. 2015).

The only detected putative species, PS3, in medium-sized lemurs (*Eulemur, Hapalemur, Prolemur*) is compatible with previous surveys in Ranomafana which found identical "strongylid" eggs in several medium-sized lemurs (Hogg 2003, as cited in Irwin and Raharison (2009)). The number of putative species in black rats is comparable to previous studies of rodents in Ranomafana National Park (Lehtonen, unpubl.): PS1 and PS3 match previously detected *Strongyloides ratti* and *Nippostrongylus brasiliensis*. We found, however, fewer species than Raharivololona *et al*. (2007) did in Mandena where they identified 15 morphospecies across 36 samples. To assess the match between coproscopy and sequencing, we dissected black rats and morphologically identified their parasites as *Nippostrongylus* sp. and *Mastophorus* sp. *Nippostrongylus* sp. positive rats had PS3 also in the larval samples, though one of the larval samples yielded a different identification, PS1. To get a conservative estimate of species richness, we excluded any OTUs that were identified as soil nematodes and were exclusively found in samples known to have come in contact with the cage floor. There were two species (PS2: *Caenorhabditis*, PS6: *Panagrellus*), which had their closest match to soil nematodes but which were also present in the samples which were not in contact with the soil or trap floors, i.e., samples collected directly from defecating animals. Furthermore, Baermann's method only allows for detection of living nematodes, which rules out nematode detection through geophagy or other accidental ingestion, which means that the possibility of these species being free-living is small. As these putative species were encountered in several species, it is possible that they are composed of several actual species, some of which are soil nematodes. In the future, expanded genetic databases could resolve, which species these putative species actually belong to.

Baermann's method is inherently limiting with respect to nematode communities resolution as

not all nematode parasite species have free-living larval forms. For example, we were unable to detect *Mastophorus* sp., a large-sized nematode that inhabits the stomach of rodents, in the larval samples. Indeed, *Mastophorus* does not have free-living larvae and could not be isolated by Baermann's method. Endemic rodents can also carry *Mastophorus* as an earlier survey found it in both endemic rodent genera (Jukka T. Lehtonen, unpubl.). Also *Enterobius* should not be detected by Baermann's method as eggs are infectious without a free-living larval stage. We suspect that the low prevalence of *Enterobius* represents chance amplifications of *Enterobius* genetic material and thus underestimates the total prevalence. An alternative method would be to isolate parasite DNA from the feces as in Tanaka *et al*. (2014), but this in turn could lead to difficulties distinguishing actual parasites inhabiting the gastrointestinal tract and accidentally ingested parasites, for example, from the diet. Irrespective,of what fecal analysis methods is used, they can only detect helminths when they are laying eggs.

The specificity of assigned labels varied depending on the nematode clade. For example, the only *Enterobius* sequence in the SILVA database would form its own cluster, i.e., it can be distinguished from all the other nematode species in the database (Table 2). In comparison, clusters based on nematode sequences from Rhabditidae or Chromadorea can contain several different species. It should be noted that this is predominantly a problem for labelling these clusters: within these taxa, there can be a high number of different clusters, i.e., they can be differentiated from each other, but they are still labelled as Rhabditidae and Chromadorea. Although there are almost 19,000 18S sequences in Genbank, from over 4,600 different species of nematode (as of February 2017, excluding environmental and metagenomic data), our samples rarely got perfect matches (Table 2). This is unsurprising as there are very few

446 sequences for intestinal nematodes from Malagasy animals published. It is also probable that
447 our analyses contain species that have not been previously described. Nevertheless, the lowest
448 common taxonomical ancestor of top scoring BLAST hits is a valid and practical way of
449 labeling putative species. Obviously, future work will be required to determine the exact
450 relationships between the nematode OTUs shared by the endemic and non-endemic hosts.
451
452 This study also demonstrates the challenge of choosing the target region for a barcoding
453 analysis: PCR amplification with universal primers requires regions with high sequence
454 conservation whereas high overall conservation limits resolution for identification on lower
455 taxonomic levels (Powers et al. 2011). The primers with a more informative target region or
456 longer amplicons could enhance the resolution of the method. One concern for the
457 metabarcoding approach is that the success of amplification and sequencing was quite low
458 (Table 1). This could be due to low levels of DNA, the nematode cuticle or the presence of
459 inhibiting substances in fecal samples. We do not believe the low success rate is due to our
460 primers systematically failing to amplify some nematode species as the success rate for the
461 second attempt of isolation and amplification for failed samples was comparable to the first
462 (30% vs. 22%). This low success rate, though, implies that using fecal parasite DNA, i.e.,
463 larvae or eggs, for DNA isolation could pose additional challenges for metabarcoding parasite
464 communities.
465
466 In conclusion, metabarcoding is a promising approach for non-invasive survey of intestinal
467 parasites. Nevertheless, our approach was limited by Baermann's method and low resolution
468 of the 18S marker gene. There is also a need for more robust DNA isolation methods to
469 ensure successful amplification. Further development could make this a useful tool for

assessing parasite communities more holistically in threatened host communities. Our results show that well-sampled host species had differing parasite assemblages and both sampling site and year affected parasite assemblages. Though there was an overlap of putative species in sympatric host species, we cannot conclude whether these are same or different parasite species.

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