Basic and Applied Herpetology 32 (2018) 29-40

Exploring non-invasive sampling of parasites by metabarcoding gastrointestinal nematodes in Madagascar frog species

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Received: 09 November 2017; returned for review: 05 February 2018; accepted: 28 March 2018.

The diversity of Anuran parasites is poorly surveyed, despite arguably being one of the most important threats to anuran populations worldwide. Additionally, parasites also interact with a number of other stressors, such as invasive species, pollution, sedimentation and changing light conditions, caused by anthropogenic disturbance in natural habitats. We aimed to explore the use of metabarcoding, a new, non-invasive tool to survey the parasite assemblages in frogs in different environments facing different levels of anthropogenic pressure. We collected fecal samples from frogs across three different transects in Ranomafana National Park, located in southeastern Madagascar, and then used the 18S metabarcoding technique to identify nematode species from the collected fecal samples. We were able to find four different putative species, which were all identified to the genus level. In comparison to the literature on previous surveys done with traditional methods, the metabarcoding approach seems to provide similar diversity estimates and taxonomical accuracy. Our results suggest that non-invasive sampling and metabarcoding can provide a suitable tool for intestinal parasite surveys in anuran host populations.

Key words: fecal egg count; helminths; *Mantidactylus*; parasite prevalence; *Ptychadena*.

Amphibian populations are declining rapidly worldwide due to anthropogenic causes such as habitat loss, pollution, collection for the pet trade, introduction of invasive species and the spread of disease (Stuart *et al*., 2004; McCallum, 2007; Sodhi *et al*., 2008). As one of the global biodiversity hot spots, Madagascar is home to over 300 described species of frogs, 99% of which are endemic (Glaw & Vences, 2007; Rakotoarison *et al*., 2017). Madagascar has thus far escaped the major amphibian biodiversity loss (Andreone *et al*., 2005) seen in many other tropical regions globally. Although recently the chytrid fungus *Batrachochytrium dendrobatidis* has been de-

DOI: https://dx.doi.org/10.11160/bah..92

scribed within the anuran populations of Ranomafana National Park (BLETZ *et al.*, 2015), there have been no reported chytrid -attributed deaths of amphibians in Madagascar. Nevertheless, with the amount of extreme habitat loss across Madagascar, including the loss of 90% of Madagascar's eastern rainforests, it is important to assess the status of Madagascar's amphibian fauna and to understand what effect anthropogenic activity is having on the amphibian species (ANDREONE *et al.*, 2008).

Anthropogenic activity can influence host-parasite interactions by introducing new parasite species to the system, changing the population dynamics of host species and affecting resistance and tolerance of individual frogs towards parasites (MCKENZIE, 2007; KING *et al.*, 2007; KOPRIVNIKAR & REDFERN, 2012; COMAS *et al.*, 2014). Thus it is necessary to survey parasite assemblages in environments with different levels of anthropogenic influence (KOPRIVNIKAR *et al.*, 2012). Indeed, there have been recent parasitological surveys on Malagasy frogs on monogeneans (VERNEAU *et al.*, 2009; DU PREEZ *et al.*, 2010; RAHARIVOLOLONIAINA *et al.*, 2011; BERTHIER *et al.*, 2014), mites (WOHLTMANN *et al.*, 2007) and protists (DELVINQUIER *et al.*, 1998), while nematodes were surveyed broadly in 1950s and 1960s (CHABAUD & BRYGOO 1957, 1958; CHABAUD *et al.*, 1961, although see recent information in KUZMIN *et al.*, 2013).

Parasitological surveys have traditionally been based on morphological identification of adult parasite stages (AHO, 1990). In the case of helminths, these stages reside within the frogs' gastrointestinal tract and thus their identification requires lethal sampling. Lethal sampling is not suitable for many situations, including wildlife health surveys of endangered species. Furthermore, non-lethal methods would be preferable over lethal sampling if the efficacy of both methods were comparable.

Fecal analysis has been used for non-invasive gastrointestinal parasite assessments, but this is rarely done in amphibians. Usually the identification of helminths based on stages in feces (i.e. eggs and larvae) is very difficult and the identification is usually done at high taxonomic levels. Nevertheless, new molecular methods could make this identification easier. Barcoding is the method of choice for molecular identification of organisms (HEBERT *et al.*, 2003), and high-throughput sequencing has created potential for sequencing simultaneously several barcode sequences (i.e. several different organisms) from a single sample. This approach is referred to as 'metabarcoding' (TABERLET *et al.*, 2012; AIVELO & MEDLAR, 2018).

Our aim in this study was to explore the suitability of non-invasive metabarcoding methods to determine intestinal parasite prevalence and diversity in several species of frogs in Madagascar. While several studies have evaluated the usefulness of non-invasive molecular sampling of parasites in reptiles (JONES *et al.*, 2012; JORGE *et al.*, 2013), there remains a lack of research on its usefulness with amphibians, most notably frogs (though see HUGGINS *et al.*, 2017).

Materials and Methods

We sampled frogs at four sites in Ranomafana National Park (RNP, 21°16′ S, 47°20′ E) in southern central east region of

Madagascar (as per BROWN *et al*., 2016). RNP consists of 43 500 hectares of lowland to montane rainforest, ranging between 500 and 1500 meters elevation, as well as a peripheral zone with limited protection (WRIGHT & ANDRIAMIHAJA, 2002). We chose sampling sites in order to sample an array of anthropogenic disturbance categories to reflect different scenarios of parasite occurrence: sites A1 and A2 were streams within the protected secondary forest, site B was a stream next to rice paddy fields and site C was a stream located downstream of site B and next to a small village. Sites A1 and A2 are subject to a low level of anthropogenic disturbance, though they are still affected by anthropogenic disturbance in the forms of tourist activity, possible illegal mining and logging and large scale environmental disturbance such as climate change (WRIGHT *et al*., 2014). Site B has a moderate level of anthropogenic disturbance in the form of agricultural activity and runoff, bank degradation and forest fragmentation. Site C has a high level of anthropogenic disturbance, as it faces an area of agricultural disturbance which also affects site B, as well as increased levels of pollution, extreme bank degradation and an influx of waste from humans and domesticated animals.

We collected frogs from each of the four sites two to three times over a period of ten days. At the beginning of each sampling, a goal was set to collect ten to fifteen frogs from the stream or within 1 meter of the stream's edge. Collection was non-discriminative and we caught all observed frogs regardless of sex, size or species. Using visual encounter methods over a period between one and two hours, we caught frogs by hand and placed them individually in small plastic bags (10 x 15 cm) containing water with a depth of one centimeter, and the rest of the volume filled with air and sealed. Frogs from sites A1 and A2 were taken to the research station within the National Park and frogs from sites B and C were taken to the lab at Centre Val-Bio. In the lab, we identified and photographed each frog and measured their length (snout to vent). We left the frogs in plastic cups lined with moist paper towels and covered with a vented plastic lid for 20 hours in the lab. After 20 hours, we collected and weighed fecal samples and released the frogs back to the site from which they were caught. We followed American Society of Ichtyologists and Herpetologists' Guideline for Use of Live Amphibians and Reptiles in Field and Laboratory Research and complied with Malagasy and local regulations. We obtained permits for sampling from both trilateral commission (CAFF/CORE) and Ranomafana National Park.

The protocol for parasite isolation and identification is described in detail in AIVELO *et al*. (2018). In short, we isolated nematodes with a modified version of Baermann's method (BAERMANN, 1917): we wrapped the fecal sample in a small piece of lab tissue (Kim-Wipe) and left the parcel suspended in filtered water in a microcentrifuge tube for 24 hours. To quantify fecal egg counts (FEC), we counted the amount of nematode larvae present in the water divided by the weight of the feces and calculated eggs per gram of feces. Then we lysed the nematode cells with proteinase K and isolated the DNA by using isopropanol precipitation. We metabarcoded the

nematode larvae by using the small subunit of ribosome (18S) gene: we amplified the marker gene with primers by BHADURY & AUSTEN (2010): M18F: 5′-AGRGGTGAAATYCGTGGAC-3′ and M18R: 5′-TCTCGCTCGTTATCGGAAT-3′. The PCR program had initial denaturation at 98°C for two minutes, then 30-40 cycles of 15 second denaturation at 98°C, annealing at 53°C for 30 seconds and 30 second extension at 72°C with 10 minutes of final elongation at 72°C. Amplicons were sequenced at the DNA Sequencing and Genomic Laboratory, Institute of Biotechnology, University of Helsinki, using a Roche 454 Genome Sequencer FLX+.

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 (i.e. reduce sequence noise produced by sequencing) each sample and then discarded sequences with ambiguous base calls: more than one error in the multiplexing barcode or more than two errors in the primer sequence. 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 five. We performed the clustering of the sequences with a similarity threshold of 99%. For labelling, we performed a MegaBLAST (CAMACHO *et al.*, 2009) search of the NR (non-redundant) database at NCBI on the cluster centroid sequences. We report the lowest common ancestor from the NCBI taxonomy of all top scoring BLAST hits (i.e. the taxon which contains all the taxa representing the top hits). We removed all operational taxonomic units (OTUs) with taxonomic labels to phyla other than Nematoda. To reduce the number of spurious OTUs, we manually combined some of the OTUs to create putative species with the following criteria: a) the OTUs were labelled to the same taxon, b) they were a monophyletic group, c) there was one dominant OTU within the group, and d) the OTUs occurred in the same individual.

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.1309923).

We performed all statistical tests and their visualizations in R using the stats package (R CORE TEAM, 2013). To model the effect of variables (date, host species, host length, sampling transect) to FEC and nematode presence-absence, we used generalized linear models (GLMs). Link functions were chosen based on the lowest residual deviance. For FEC analysis, to account for the high over-dispersion in our data, we used negative binomial error structure and log link function. In addition, we explored the FEC by first van der Walden transforming. For nematode presence modelling, we used GLM with binomial error structure with complementary log-log link function. For each of the models, we initially included all variables with their interactions and dropped non-significant interactions and variables sequentially as long as the resulting new model had lower values for Akaike's information criterion.

Table 1: Number of frog samples by species (N), including number of frogs that produced feces (Feces), number of fecal samples containing nematodes (Nemat.), number of those nematode samples that were successfully sequenced (Seq.), and the detected putative species (PS), including their labels .

Frog species	N	Feces	Nemat.	Seq.	PS1: *Strongyloides*	PS2: *Caenorhabditis*	PS3: *Rhabditoides*	PS4: *Raillietnema*
Mantidactylus lugubris	48	25	14	4	1	0	3	2
Ptychadena mascarensis	43	30	24	8	2	3	3	6
Mantidactylus grandidieri	1	0						
Gephromantis tschenki	1	1	0					
Mantidactylus alutus	1	1	0					
Mantidactylus femoralis	1	1	0					
Not identified	8	0						

RESULTS

We caught a total of 103 frog individuals belonging to at least seven frog species (Table 1). Fifty-nine individuals produced feces during their 20 hours in captivity varying from 0.01 to 0.11 grams. Thirty-nine individuals (66%) had nematodes extracted from their fecal samples with FEC from 22 to 7700 eggs per gram feces. We were successful in sequencing only 31% of the samples.

We had a total of 40 513 reads, which were reduced to 22 161 reads after quality control. We were able to find four nematode putative species. The median number of high quality reads representing putative species per sample was 1794 with an interquartile range of 1580-2059. The four putative species were labelled to the genus level (Table 1). One of the putative species matches to free-living nematode genera (PS2: *Caenorhabditis*), but we included it as the cluster is the same OTU as previously identified in mouse lemur samples which could not have been contaminated with environmental contamination (AIVELO *et al*., 2015).

As is usual with parasite data, our data was heavily zero-inflated. Thus we used all samples for which we had feces for modelling parasite presence, but then only used samples containing nematodes to model FECs. When FEC model residuals were plotted against fitted values, the data appeared to be homoscedastic. When comparing the FEC model with non-transformed FECs and FEC transformed to van der Waerden scores, non-transformed FECs had lower residual deviance and they were used in the final model. We did notice a strong collinearity between frog length and host species (*Mantidactylus lugubris* was significantly larger than *Ptychadena mascarensis*; mean ± SD: 36.8 ± 14.26 vs. 27.0 ± 9.28 mm, respectively; t_{81} =

Table 2: Coefficients from the final models of nematode presence and fecal egg counts (FEC). For both models, transect B had statistically significant difference to transect A.

Model	Coefficients	Estimate	Std. error	Z	*P*
Nematode presence ~ transect + length	Intercept	-1.13	0.82	-1.38	0.17
	Transect B	1.07	0.53	2.03	0.04
	Transect C	0.96	0.50	1.93	0.05
	Host size	0.02	0.02	1.11	0.27
FEC ~ transect + length	Intercept	5.79	0.89	6.53	<0.001
	Transect B	1.26	0.56	2.25	0.02
	Transect C	0.70	0.52	1.35	0.18
	Host size	0.02	0.02	1.24	0.21

Table 3: Comparison of encountered parasite diversity in our non-invasive metabarcoding study in comparison with previous literature using traditional methods.

Studied system	Number of host spp.	Number of nematode spp.	Mean number of nematodes per host spp.	Sample size	Reference
Malagasy rainforest frogs	2	4	3.5	91	This study
Microlophus lizards in Peruvian rainforest	7	5	2.0	75	Goldberg & Bursey (2009)
Amphibians in savannah in Benin	14	8	1.1	145	Aisien *et al.* (2011)
Leptodactylus in NE Argentina	1	6	6.0	76	Hamann *et al.* (2012)
Tropidurids in NE Brazil	4	3	1.5	24	Lambertz *et al.* (2012)
Rana pipiens in Quebec, Canada	1	5	5.0	146	Shutler *et al.* (2015)
Anurans in Brazil pantanal	5	19	6.0	120	Campião *et al.* (2017)

3.90, $P < 0.001$) and thus only one of these variables were included in final model. We did not find evidence of overfitting in the models.

For both nematode presence and FEC models, the final model included transect and host size as the variables, of which transect B was statistically significant (Table 2). We dropped frog species and collection date variables and all interactions from the models.

As the sample size for the identified nematode species is low, we can only do limited analysis with the species patterns. Two of the species were rather rare: both PS1 (*Strongyloides* sp.) and PS2 (*Caenorhabditis* sp.) were present in three samples. In addition, PS1 had very low amplicon counts (12-20 amplicons). PS3 (*Rhabditoides* sp.) and PS4 (*Raillietnema* sp.) were more common, with presence in six and eight hosts, respectively. For host species, half of the individuals were co-infected with two or three putative parasite species.

Table 4: Nematode prevalence and fecal egg counts (FEC) for two of the most common frog species in different sampling transects. Frog size is measured as snout-to-vent length in millimeters, while fecal egg counts are nematode larvae per gram of feces. Both variables are given as median values with interquartile range in parentheses mentioned in text.

Site	Habitat	Frog species	N	Frog size	Nematode prevalence (%)	FEC
A	Secondary forest	*M. lugubris*	40	38 (19)	56	625 (1600)
		P. mascarensis	3	32 (3.5)	0	
B	Rice paddies	*M. lugubris*	1	13 (-)	100	100 (-)
		P. mascarensis	25	23 (18)	78	1360 (2560)
C	Downstream from a village	*M. lugubris*	7	25 (15)	50	1290 (625)
		P. mascarensis	15	27 (14)	91	937 (1990)

Discussion

We successfully used non-invasive sampling with metabarcoding to identify parasite assemblages in sympatric Malagasy frog species. We did not directly compare efficiency of traditional approaches to amphibian parasite surveys, that is, dissecting the intestines and morphologically identifying adult nematodes and that of our method. Nevertheless, previous studies have found comparable parasite richness in frogs (Table 3). *Strongyloides* sp., *Raillietnema* sp., and *Rhabditoides* sp. are all common parasites of anurans (Bursey & Goldberg, 2006; King *et al*., 2010; Jones *et al*., 2012; Shutler *et al*., 2015). Thus, our sampling seems to give a reliable estimate of parasite community composition and our labelling seems to be a largely reliable way of identifying the putative species (Table 1), though further validation is required.

We sampled frogs in different environments to test our method with frogs which could be expected to have different parasite communities and differences in parasite diversity. While the parasite communities were highly similar, there were differences in likelihoods of parasite infection and fecal egg counts (Table 2). Frog species did not occur uniformly as *P. mascarensis* was more common in disturbed areas, while *M. lugubris* was more common in selectively logged forest (Table 4). Previous research has shown that while frog assemblages differ between continuous forests, fragmented forests and matrix streams, the overall species richness is similar among these habitats (Riemann *et al*., 2015; Ndriantsoa *et al*., 2017), thus suggesting that species diversity is not the main factor driving differences among sites, but it can partly explain why there were not large differences. We were not able to model the difference in parasitism between host species, as it correlated strongly with frog size, and the number of individuals per species was too low to get robust results from modelling species separately. We also expect that frog size is correlated with the age or body condition of frog individuals, both of which could have a strong influence on the parasite dynamics and on the possibility of acquiring or clearing parasite infection (Comas *et*

al., 2014).

Metabarcoding is hindered by the same general problems as fecal sampling (AIVELO & MEDLAR, 2018). Fecal sampling can be used to detect only those parasites which are currently laying eggs in the intestine (GILLESPIE, 2006). Furthermore, adult nematode numbers can be used to assess parasite loads, but with fecal sampling the infection intensity has to be assessed with proxies. Fecal egg counts have been shown to be quite unreliable and poorly repeatable (STEAR *et al*., 1995; WOOD *et al*., 2013), though widely used due to the lack of alternatives for non-invasive sampling.

Our approach had a rather low success rate for amplifying nematode DNA from anuran feces. This could be due to low amounts of DNA in the sample, the protecting nematode cuticle or the presence of inhibitors in fecal samples. This in turn leads to the conclusion that our analysis is limited by the number of samples, especially by the number of successfully sequenced samples; a little over half of the caught amphibians produced fecal samples, of which a little over half had nematode parasites, which in turn were successfully sequenced in only approximately one third of the cases (Table 1).

Barcoding has been proven to be especially difficult in nematodes (BHADURY & AUSTEN, 2010; POWERS *et al*., 2011). While the cytochrome oxidase I gene is usually used in metazoan barcoding, it does not work well with nematodes as there is a lack of universal primers. Thus, the most common marker gene is 18S, which is normally used to separate taxa at high taxonomic levels (PORAZINSKA *et al*., 2010). Nevertheless, for the parasites present in our sample, this marker gene and our primers worked well in identifying the species to the genus level. More accurate labelling would require more extensive databases: while almost 5000 nematode species are represented in GenBank, this is still only a small proportion of all described nematodes. As we did not get close matches, the detected nematodes likely were species which are not yet available in public databases.

In conclusion, our work shows that metabarcoding is a promising new technique that could be used to non-invasively assess frog gastrointestinal parasites. More work is still required in validating the fecal collection methods, improving sequencing success and strengthening species labelling accuracy. Metabarcoding can prove to be especially useful in frog populations which are endangered or in situations when taxonomic expertise of nematodes is lacking.

Acknowledgement

We would like to thank the Madagascar Ministry of Environment, Ecology and Forests, as well as the Madagascar National Park Service for giving us permission to conduct this research in Madagascar. The authors wish to thank Ricardo Rocha on comments on the manuscript, Tharcisse Ukizintambara for coordinating the Stony Brook University Madagascar Study Abroad course at Stony Brook University, the Centre Valbio research station staff for logistical and infrastructural support, James Herrera for his guidance in the field, Raija Savolainen and Agnes Viherä for help in laboratory analysis and Alan Med-

lar for help in bioinformatical analysis. This work is based on an individual study project by K.H., who planned the project and collected the samples under the supervision of T.A., J.C., and P.W. T.A. performed the metabarcoding and statistical analysis. The manuscript was written by T.A. and K.H. with all authors commenting on the drafts.

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