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Pacific Conservation Biology

Supplementary Material

Evaluating the utility of environmental DNA for detecting a large Critically Endangered lizard in tropical northern Australia

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Supplementary Material S1

Table S1. Samples collected for eDNA analysis in October 2022, including the activity levels (based on the number of camera detections prior to surveys) of the target species, the Arnhem rock skink (*Bellatorias obiri*). Rows are coloured by activity level.

Sample	Location	Type	Activity Level	# samples collected	Date collected
B01	Main Gorge	Soil	High	2	17/10/2022
B03	Main Gorge	Soil	High	2	17/10/2022
B23	Upper Gorge	Soil	High	2	17/10/2022
B26	Upper Gorge	Soil	High	2	17/10/2022
B16	Upper Gorge	Soil	Low	2	17/10/2022
B18	Upper Gorge	Soil	Low	2	17/10/2022
B22	Upper Gorge	Soil	Low	2	17/10/2022
B30	Upper Gorge	Soil	Low	2	17/10/2022
B08	Lower Gorge	Soil	None	2	17/10/2022
B09	Lower Gorge	Soil	None	2	17/10/2022
B14	Lower Gorge	Soil	None	2	17/10/2022
B15	Lower Gorge	Soil	None	2	17/10/2022
B05	Main Gorge	Scat	Low	1	5/9/2022
Main Pool	Main Gorge	Water	-	2	17/10/2022
Upper Pool	Upper Gorge	Water	-	2	17/10/2022
Lower Pool	Lower Gorge	Water	-	2	17/10/2022

Figure S1. Collecting soil samples from crevices occupied by Arnhem rock skinks for environmental DNA analysis. (Photo credit: Georgia Kielbaska).

Table S2. Species detected via camera traps facing 12 rock crevices between 1 July and 9 September 2022, prior to soil sampling in October 2022. Bold indicates species that were detected via eDNA metabarcoding of soil or water samples. *Bellatorias obiri* was detected via eDNA from a scat but not soil or water.

Class	Family	Species	Total number of photos
Amphibia	Bufonidae	***Rhinella marina***	78
	Hylidae	***Litoria* sp.**	3
Mammalia	Dasyuridae	*Planigale maculata*	12
		Pseudantechinus bilarni	1162
	Felidae	*Felis catus**	9
	Macropodidae	***Osphranter bernardus***	759
		Petrogale wilkinsi	431
	Muridae	*Hydromys chrysogaster*	105
		*Rattus rattus**	96
		Zyzomys argurus	1213
	Tachyglossidae	*Tachyglossus aculeatus*	15
		Unknown small mammal	79
Squamata	Colubridae	*Dendrelaphis punctulatus*	29
	Elapidae	*Pseudechis weigeli*	13
	Pythonidae	*Antaresia childreni*	3
		Liasis olivaceus	24
	Scincidae	*Bellatorias obiri*	104
		Ctenotus sp.	62
		Morethia ruficauda	244
		Unknown skink sp.	3
	Varanidae	*Varanus glebopalma*	175
		Varanus insulanicus	35
		Unknown snake sp.	16
		Unknown gecko sp.	6

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Supplementary Material S2

Sample preparation, DNA extraction and library preparation

Prior to sample processing, GenBank was queried for reference sequences for the target species, *Bellatorias obiri*, to determine the most appropriate molecular technique to apply to the samples. No reference sequences currently exist for *B. obiri*; as such, a species-specific qPCR assay could not be designed for use in this study. Reference sequences do exist for some closely related species (genera *Bellatorias*, *Egernia*, *Liopholis*), which allowed *in silico* testing of the 16S Reptile metabarcoding assay of West *et al* (2023) to be performed as an indicator of the likelihood of the assay being able to successfully amplify the target. No base-pair mismatches were found between the assay and the tested species, suggesting it would be suitable for detecting *B. obiri*. Additionally, sufficient genetic variation existed between all tested species to suggest that this assay would be appropriate for discrimination between skinks in the region. To ensure the assay successfully amplified the target *in vitro*, a tissue specimen was obtained from the Museum and Art Gallery of the Northern Territory (Museum Sample ID: TS0052) and used as a positive control sample to ensure the assay successfully amplified the target species.

DNA was extracted from the tissue sample and half of each filter paper using a Qiagen DNeasy blood and tissue kit with a modified protocol (540µL of ATL and 60µL ProK were used), and from an approximate 250 mg sub-sample of the soil and scat samples using a Qiagen PowerSoil Pro kit (as per Koziol *et al*. 2019; Stat *et al*. 2017, 2019). Extractions were performed using an automated QIAcube extraction platform (Qiagen). All extractions were undertaken in a dedicated PCR-free laboratory, and extraction controls processed alongside samples. Extractions were eluted in a final volume of 100 µL AE buffer.

To determine the required dilution for optimal amplification, PCR reactions were performed in duplicate on each extraction by adding DNA template directly to the PCR master mix (neat), then performing a serial dilution (1 in 10). The PCRs were performed at a final volume of 25 µL where each reaction comprised of: 1 × PCR Gold Buffer (Applied Biosystems), 0.25 mM dNTP mix (Astral Scientific, Australia), 2 mM $MgCl_2$ (Applied Biosystems), 1U AmpliTaq Gold DNA polymerase (Applied Biosystems), 0.4 mg/mL bovine serum albumin (Fisher Biotec), 0.4 µM forward and reverse primers (forward 5′-AGACNAGAAGACCCTGTG-3′, reverse 5′-CCTGATCCAACATCGAGG-3′; West et al., 2023), 0.6 µl of a 1:10,000 solution of SYBR Green dye (Life Technologies), and 2 µL

template DNA. PCRs were performed on StepOne Plus instruments (Applied Biosystems) with the following cycling conditions: 95 °C for 5 min, followed by 50 cycles of: 95 °C for 30 sec, 52 °C for 30 sec, 72 °C for 45 sec, then a melt-curve analysis of: 95 °C for 15 sec, 60 °C for 1 min, 95 °C for 15 sec, finishing with a final extension stage at 72 °C for 10 min. All reactions for the positive control tissue sample showed amplification, confirming that the assay would be able to successfully amplify the target DNA.

After selection of the optimal dilution (neat or 1 in 10), PCRs were repeated in duplicate as described above but instead using unique, single use combinations of 8 bp multiplex identifier-tagged (MID-tag) primers as described in Koziol *et al*. (2019) and van der Heyde *et al.* (2020). Master mixes were prepared using a QIAgility instrument (Qiagen) in an ultra-clean lab facility, with negative and positive PCR controls included on every plate to ensure the validity of results. A sequencing library was created by combining samples into mini-pools based on the PCR amplification results from each sample. The mini-pools were then combined in roughly equimolar concentrations to form libraries. Libraries were then size selected (250-600 bp cut-off) using a Pippin Prep instrument (Sage Sciences) with 2% dye-free cassettes, cleaned using a QIAquick PCR purification kit, quantified on a Qubit (Thermo Fisher), and diluted to 2nM. The libraries were sequenced on an Illumina MiSeq instrument using 500-cycle kits with custom sequencing primers.

Sequencing and bioinformatics pipeline

Raw metabarcoding sequence data was analysed using the eDNAFlow pipeline (Mousavi-Derazmahalleh *et al.* 2021), where data were demultiplexed and trimmed using Obitools and quality filtered with Usearch v11 for sequencing errors (maxee=1) with custom filtering parameters applied (--minAlignLeng '12', --minLen '70', --minsize '2'). Sequences were transformed into zero radius operational taxonomic units (ZOTUs) to provide sensitive taxonomic resolution. Generated ZOTUs were queried against the nucleotide database NCBI (GenBank) and assigned to the species level where possible (i.e., a match >95% similarity to a single species was provided) or dropped back to the lowest common ancestor if multiple possible taxonomic assignments were given. Taxonomic assignments were based on an in-house Python script which further filters the Blast results, combines them with the ZOTU table results and produces a table containing the taxonomic information available from Blast taxonomy database (custom parameters: --lca_pid '95', --lca_diff '0.5', --minMatch_lulu '97'). Assigned species identifications were then assessed to confirm their presence in the study region.

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