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Development of a *16S* metabarcoding assay for the environmental DNA (eDNA) detection of aquatic reptiles across northern Australia

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Abstract. A severe lack of distribution data for aquatic reptiles in northern Australia leaves many taxa vulnerable to extirpation and extinction. Environmental DNA (eDNA) technologies offer sensitive and non-invasive genetic alternatives to trapping and visual surveys and are increasingly employed for the detection of aquatic and semi-aquatic reptiles. However, these eDNA approaches have largely applied species-specific primers that do not provide a cost-effective avenue for the simultaneous detection of multiple reptilian taxa. Here, we present a mitochondrial *16S* rRNA metabarcoding assay for the broad detection of aquatic and semi-aquatic reptile species. This assay is tested on water samples collected at multiple sampling sites at two tropical locations, including 12 marine and estuarine sites in Roebuck Bay, Western Australia, and four estuarine sites in Cooktown, Queensland, Australia. In total, nine reptile taxa were detected from 10 of the 16 sampled sites, including marine and freshwater turtles, aquatic, semi-aquatic and terrestrial snakes, and terrestrial skinks. However, inconsistencies in the detection of previously observed aquatic reptiles at our sampled sites, such as saltwater crocodile and sea snakes, indicated that further research is required to assess the reliability, strengths and limitations of eDNA methods for aquatic reptile detection before it can be integrated as a broad-scale bioassessment tool.

Keywords: aquatic reptile, environmental DNA, marine turtles, metabarcoding, northern Australia, sea snakes.

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Introduction

The aquatic and semi-aquatic reptile fauna of northern Australia are highly distinctive, exhibiting life history traits and physical adaptations to extreme climates and, in the case of semi-aquatic species, seasonal, but increasingly inconsistent, water availability (Pusey 2011). Collectively, these taxa form an integral component of the region's aquatic and riparian food webs across multiple trophic levels; the saltwater crocodile (*Crocodylus porosus*) often fills the role of apex predator in these ecosystems. There are over 90 recognised aquatic and semi-aquatic reptile species in Australia, including marine and freshwater turtles, crocodiles, monitor lizards, water skinks, water dragons, sea snakes, sea kraits and other semi-aquatic snakes (P. Uetz, P. Freed, and J. Hošek, see http://www.reptile-database.org). These aquatic and semi-aquatic reptile species are under increasing threat from pollution, urban development, over-harvesting, fisheries by-catch, invasive species (such as the toxic cane toad, *Rhinella marina*), and climate change, with the latter affecting the frequency of bushfires, coastal erosion and coral reef degradation (Böhm *et al.* 2013; Doody *et al.* 2014; Milton 2001; Wilcox *et al.* 2015). In addition, latitudinal niche shifts to mitigate changing temperatures have prehistorically been much more challenging for ectotherms, such as reptiles, which have a lower climatic tolerance and reproductive ability (Rolland *et al.* 2018). The viability of reptile species that exhibit temperature-dependent sex determination may be compromised with projected temperature shifts in the next 50 years (Santidrián Tomillo *et al.* 2015). Thus, the continual monitoring of aquatic and semi-aquatic reptile species, particularly in northern Australia, is an increasing necessity.

Typical survey techniques for the detection of aquatic reptiles include snorkelling, trapping, satellite tracking, aerial surveying, seining and by-catch reports, although many of these methods can

be limited in northern Australian waterbodies by the threat posed by saltwater crocodiles (Australian Government 2011). In comparison to other faunal groups, there is a severe lack of data on aquatic reptile distributions across catchments in northern Australia (Fox 2008). The majority of aquatic reptile surveys in this region have targeted the saltwater crocodile; two thirds of consolidated distribution records of aquatic and semi-aquatic reptiles are attributed to this species (Fox 2008), leaving data deficiencies for other reptilian taxa. This includes sea snake species, such as the Arafura sea snake (*Aipysurus tenuis*), Zweifel's sea snake (*Enhydrina zweifeli*) and the northern mangrove sea snake (*Parahydrophis mertoni*; Elfes *et al.* 2013). Other data-deficient (DD) aquatic reptile taxa in northern Australia include freshwater turtles, such as Irwin's snapping turtle (*Elseya irwini*), the Gulf snapping turtle (*Elseya lavarackorum*), and the northern yellow-faced turtle (*Emydura tanybaraga*; Van Dyke *et al.* 2018), and the marine flatback turtle (*Natator depressus*).

Although a high level of data deficiency does not necessarily correspond directly to an elevated extinction risk, insufficient information in regard to population trajectories, distribution and taxonomy creates a lot of uncertainty around extinction risk, conservation priorities and legislation (Böhm *et al.* 2013; Bland and Böhm 2016). Robust temporal and spatial distribution records provide the baseline on which species ecology and conservation status can (and must) be developed. However, given the sheer number of DD reptile taxa that may or may not be threatened, it remains economically challenging to conduct in-depth surveys. Furthermore, DD taxa may require more specialised trapping techniques, taxonomic expertise, and may never be detected in timeframes that are required for management decisions, such as in relation to coastal development assessments.

Environmental DNA (eDNA) technologies offer a sensitive, cost-effective and non-invasive genetic alternative to individual species and multi-taxon surveying in marine, freshwater and terrestrial environments (Thomsen *et al.* 2012; Bohmann *et al.* 2014; Evans *et al.* 2016; Olds *et al.* 2016). Reptile eDNA studies have largely applied species-specific markers to amplify individual species from mixed environmental samples (Davy *et al.* 2015; de Souza *et al.* 2016; Halstead *et al.* 2017; Feist *et al.* 2018; Baker *et al.* 2020; Ratsch *et al.* 2020; Rose *et al.* 2020). The first reptile eDNA study, published in 2014, developed a diagnostic polymerase chain reaction (PCR) assay to detect the Burmese python (*Python bivittatus*), a semi-aquatic, invasive species in Florida (Piaggio *et al.* 2014). The python's elusive nature, cryptic colouration and occupation of aquatic habitats that were logistically difficult to survey prompted an eDNA approach. Piaggio *et al.* (2014) developed a *P. bivittatus*-specific mitochondrial cytochrome *b* assay that was applied to water samples from field sites in southern Florida, successfully detecting the species where it had been previously observed.

Advances in high-throughput sequencing now allow the simultaneous amplification and sequencing of multiple taxa through universal or broad-taxonomic PCR assays (referred to as eDNA metabarcoding), proving to be a more efficient approach to genetic surveying. However, the use of eDNA metabarcoding for the detection of reptile assemblages has not yet been thoroughly tested. Kelly *et al.* (2014) applied vertebrate-specific mitochondrial *12S* rRNA primers to detect green sea turtle (*Chelonia mydas*) in a large mesocosm, but were unsuccessful, despite successfully amplifying the intended target with species-specific primers. Conversely, Lacoursière-Roussel *et al.* (2016) had more success in using cytochrome c oxidase I (*COI*) metabarcoding assays to detect three species of snake and two species of turtle across lakes and rivers in Canada. Our primary objectives in this study were to design a metabarcoding assay that is able to simultaneously target aquatic and semi-aquatic reptile groups in northern Australia and to test its utility on water samples collected across northern Australia.

Materials and methods

Field sampling

In total, twenty-two 1-L surface-water samples were collected at 12 sites in Roebuck Bay, Western Australia, in August 2018 and twenty 1-L surface-water samples were collected at four sites near Cooktown, Queensland, in March–April 2020 (Fig. 1, Table S1 of the Supplementary material). Roebuck Bay is a semiarid, tropical, marine embayment characterised by intertidal sand, mudflat and mangrove habitats. Surface-water samples were collected during the dry season at eight ocean sites, two estuarine creek sites, and two intertidal mangrove sites. Several marine turtle and sea snake species were observed during sampling across the majority of sites; in addition saltwater crocodiles have been previously observed in the vicinity of the two sampled creek sites (Table S1). The Cooktown region comprises a variety of tropical landscapes such as sandy beaches, tidal estuaries, freshwater wetlands and rainforest hinterland. Surface-water samples were collected in the wet season at four estuarine creek sites that outflow into the ocean, two of which contained mangrove vegetation and two were connected to a sandy wetland system. The Cooktown surface-water samples were collected using a large pole to mitigate saltwater crocodile risks in the area. Water samples were individually filtered across Pall 0.45-μm GN-6 Metricel mixed cellulose ester membranes by using a Pall Sentino Microbiology pump (Pall Corporation, Port Washington, NY, USA). Filter membranes were immediately frozen and stored at –20°C prior and post-transportation to the Trace & Environmental DNA (TrEnD) Laboratory in Perth, Western Australia.

In silico design

Mitochondrial DNA is typically targeted for eDNA metabarcoding assays because of both template copy number and reference data. Two Indo-Pacific sea snake databases were curated for the mitochondrial *16S* rDNA and cytochrome *b* gene regions by Sanders *et al.* (2013) and provided for this project. The cytochrome *b* database was substantially larger (361 sequences, 1104-bp length) than the *16S* database (50 sequences, 531-bp length); however, the cytochrome *b* region presented no conserved regions that were consistent across all sea snake sequences and that would be suitable for primer binding. Conversely, the *16S* region exhibited a few conserved regions (~30–50-bp length) flanking larger hypervariable regions. Additional *16S* rDNA sequences of several Australian aquatic and semi-aquatic reptiles (Table 1) were downloaded from NCBI GenBank (https://www.ncbi.nlm.nih.gov/genbank/, accessed July 2020) and aligned using the MUSCLE plugin in Geneious (ver. 10.0.6, Biomatters Ltd, Auckland, New Zealand, see https://www.geneious.com/). Primer pairs were designed in conserved regions by using the built-in primer-design tool in Geneious; the final primer pair was chosen by visually inspecting alignments for a

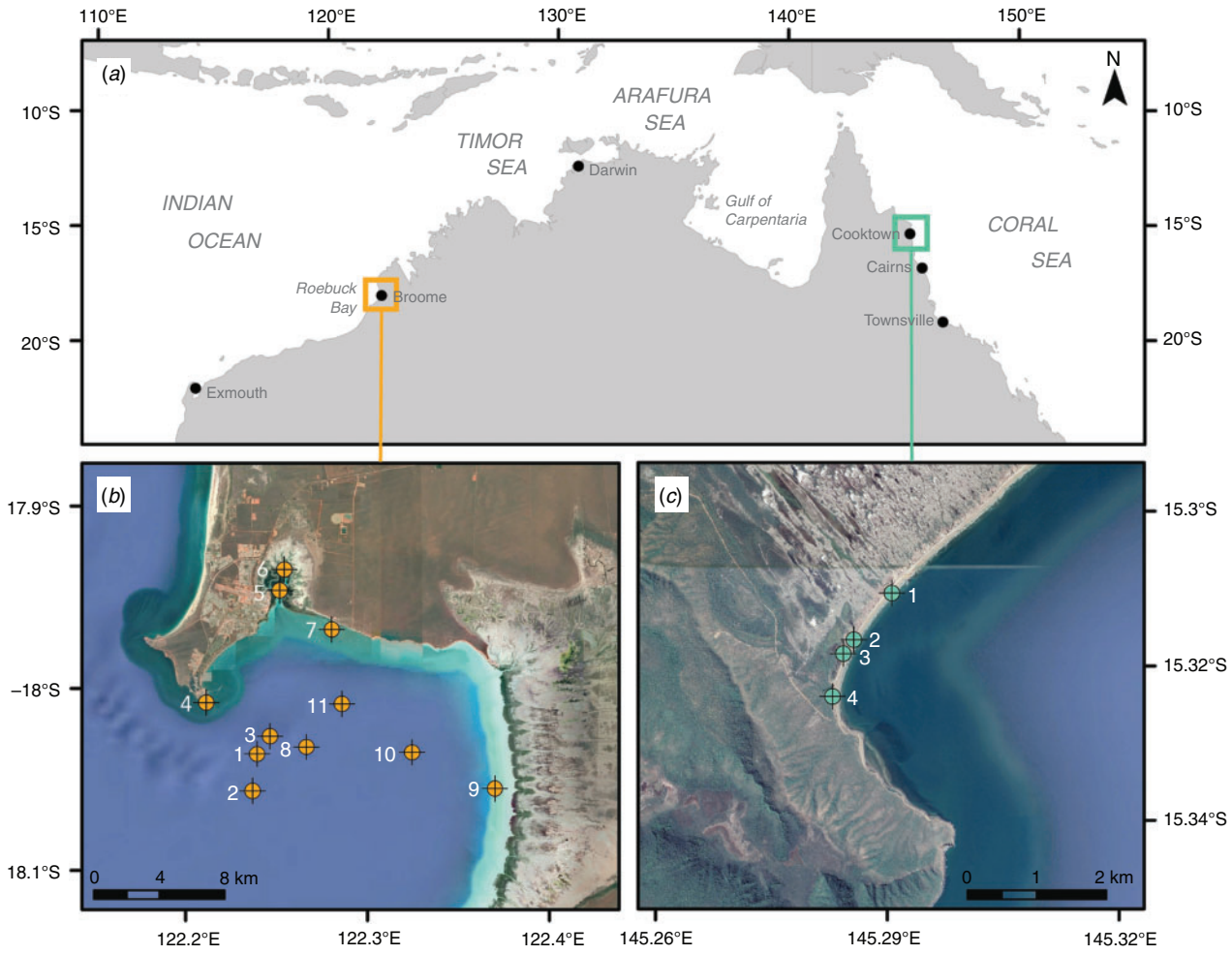

Fig. 1. (*a*) Location of sampling sites across northern Australia. One-litre water samples were collected (*b*) at 12 sites in Roebuck Bay, Western Australia ($n = 22$) and (*c*) from four sites near Cooktown, Queensland ($n = 20$). Further site information is provided in Table S1. Map data: Google Earth, SIO, NOAA, USA Navy, NGA, GEBCO; image: Landsat/Copernicus (*b*), CNES/Airbus (*c*).

target region with maximum variation and under a length of 280 bp. The length constriction allows for the sequencing of barcode indexes (on the 5′ end of both forward and reverse primers within a fusion-tagged primer setup) on a single-end Illumina MiSeq sequencing run (up to 325 bp using a 300-cycle MiSeq V2 Standard Flow Cell, Illumina, San Diego, CA, USA). The forward primer was modified to include a degenerate base, allowing for annealing to polymorphic sites in the reptile alignment. The resulting primer pair (herein referred to as the *16S* Reptile assay) is AqReptileF-degenerate: 5′-AGACNAGAAGACCCTGTG-3′ and AqReptileR: 5′-CCTGATCCAACATCGAGG-3′, with a G/C content between 50.0 and 55.6%, T_M between 52.0 and 55.5 and hairpin T_M of 32.4.

In vitro *testing on reptile tissue and eDNA water samples*

In vitro testing of the primers to assess PCR amplification and optimise the annealing temperature was first conducted using tissue extracts (1/10 dilution) from saltwater crocodile (*C. porosus*) and flatback turtle (*N. depressus*). The primers were then further tested on the filtered water samples collected at Roebuck Bay, Western Australia, and Cooktown, Queensland. DNA was extracted from half of each filter membrane using a DNeasy Blood and Tissue Kit (Qiagen, Venlo, Netherlands), with the following modifications: 540 µL of ATL lysis buffer, 60 µL of Proteinase K and a 3-h digestion at 56°C. Blank controls were processed in parallel with all samples to detect any cross-contamination. Environmental DNA extracts were then stored at –20°C.

A gradient PCR determined an optimum annealing temperature of 52°C. Each quantitative PCR (qPCR) reaction was performed in a volume of 25 µL, containing the following: 1× AmpliTaq Gold PCR buffer (Thermo Scientific, Waltham, MA, USA), 2 mM $MgCl_2$, 0.1 mM dNTPs, 0.2 µM each of forward and reverse primers (Integrated DNA Technologies, Coralville, IA, USA), 10 µg bovine serum albumin (BSA, Fisher Biotec Australia, Wembley, WA, Australia), 0.6 µL of 5× SYBR Green (Thermo Scientific), 1 U of AmpliTaq Gold DNA Polymerase (Thermo Scientific), 4 µL of template DNA, and made to volume with ultrapure distilled water (Thermo Scientific). Tissue and eDNA extracts were amplified in duplicate on a StepOnePlus Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) under the following conditions: initial denaturation at 95°C for 5 min, followed by 50 cycles of 30 s at 95°C, 52°C for 30 s and 45 s at 72°C,

Table 1. Australian aquatic and semi-aquatic reptile species targeted for *in silico* primer design and analysis
Target length refers to the length of gene. Primer mismatches indicate number of nucleotide mismatches between the reference sequence and the forward primer (F)

Common name	Species name	GenBank accession number	Target length (bp)	Primer mismatches
Sea snake				
Short-nosed sea snake[A]	*Aipysurus apraefrontalis*	JX423420	213	0
Dusky sea snake[A]	*Aipysurus fuscus*	JX423430	213	0
Olive sea snake[A]	*Aipysurus laevis*	EU547181	213	0
Turtle-headed sea snake[A]	*Emydocephalus annulatus*	EU547185	214	0
Black-headed sea snake[A]	*Hydrophis atriceps*	KC014320	212	0
Blue-banded sea snake[A]	*Hydrophis cyanocinctus*	KC014331	212	0
Ornate sea snake[A]	*Hydrophis ornatus*	KC014358	212	0
Yellow-bellied sea snake[A]	*Pelamis platurus*	KC014375	212	0
Stokes's sea snake	*Hydrophis stokesii*	JQ217146	212	0
Horned sea snake	*Hydrophis peronii*	KU323976	212	0
Shaw's sea snake	*Hydrophis curtus*	KX239662	212	0
Sea krait				
Yellow-lipped sea krait	*Laticauda colubrine*	NC_036054	212	0
Crocodile				
Saltwater crocodile[B]	*Crocodylus porosus*	NC_008143	275	0
Freshwater crocodile	*Crocodylus johnstoni*	NC_015238	275	0
Marine turtle				
Flatback turtle[B]	*Natator depressus*	NC_018550	259	0
Loggerhead turtle	*Caretta caretta*	MF579505	258	0
Olive ridley turtle	*Lepidochelys olivacea*	DQ486893	258	0
Leatherback turtle	*Dermochelys coriacea*	JX454992	259	0
Green turtle	*Chelonia mydas*	JX454990	260	0
Hawksbill turtle	*Eretmochelys imbricata*	MF571906	258	0
Freshwater turtle				
Pig-nosed turtle	*Carettochelys insculpta*	FJ862792	250	0
Cann's snake-necked turtle	*Chelodina canni*	NC_041286	254	0
Northern snake-necked turtle	*Chelodina rugosa*	KY776451	253	0
Northern snapping turtle	*Elseya dentata*	KY779844	251	0
Red-faced turtle	*Emydura victoriae*	NC_042473	253	0
Western swamp turtle	*Pseudemydura umbrina*	NC_035731	244	0
Monitor lizard				
Mangrove monitor	*Varanus indicus*	EF193674	219	1 (F)
Argus monitor	*Varanus panoptes*	EF193685	223	1 (F)

[A]Selection of reference sequences sourced from Sanders *et al.* (2013) for primer design.
[B]Species whose tissue extracts were additionally used to test primers *in vitro*.

with a final extension for 10 min at 72°C. Quantitative PCR was performed in a single step by using fusion-tagged primer architecture that comprised a forward or reverse primer sequence, a unique index (6–8 bp in length) and an Illumina-compatible sequencing adaptor. All qPCR reactions were prepared in dedicated clean room facilities at the TrEnD Laboratory, Curtin University. Quantitative PCR amplicons were pooled at equimolar ratios based on their respective qPCR ΔRn values and were then size-selected (150–600 bp) by using a Pippin-Prep (Sage Science, Beverly, MA, USA) to remove any off-target amplicons and primer dimer. Size-selected libraries were then purified using the Qiaquick PCR Purification Kit (Qiagen), quantified using a Qubit Fluorometer (ver. 4.0, Invitrogen, Carlsbad, CA, USA) and diluted to 2 nM for loading onto a 300-cycle MiSeq V2 Standard Flow Cell. Sequencing was conducted on an Illumina MiSeq platform, housed in the TrEnD Laboratory at Curtin University, WA, Australia.

Sequencing reads were demultiplexed using the ngsfilter (allowing up to three mismatches in primer sequences) and obisplit commands in the package OBITools (ver. 1.2.9, see https://git.metabarcoding.org/obitools/obitools/wikis/home; Boyer *et al.* 2016) in RStudio (ver. 1.1.423, RStudio, Inc., Boston, MA, USA). Data were then quality filtered (minimum length = 100, maximum expected errors = 2, no ambiguous nucleotides), denoised, filtered for chimeras and dereplicated (pool = TRUE) using the DADA2 bioinformatics package (Callahan *et al.* 2016) also implemented in RStudio. The resulting amplicon sequence variant (ASV) fasta file was queried against NCBI GenBank nucleotide database (accessed in July 2020) (Benson *et al.* 2005) by using BLASTn (minimum percentage identity of 90, maximum target sequences of 10, reward value of 1) in Zeus, an SGI cluster, based at the Pawsey Supercomputing Centre in Kensington, WA, Australia. Taxonomic assignments of ASVs were curated using a lowest common ancestor (LCA) approach (https://github.com/mahsa-mousavi/eDNAFlow/tree/master/LCA_taxonomyAssignment_ scripts, accessed June 2020; Mousavi-Derazmahalleh *et al.*, unpubl. data), whereby the top 10 hits for each query are

sequentially collapsed to the lowest common ancestor if the percentage identity between each consecutive hit differs by less than 1% (based on 100% query coverage). Each finalised taxonomic assignment, therefore, represents a query hit that is distinct from closely related taxa.

No permits were necessary for water sampling.

Results

Primer design

The *16S* Reptile assay was designed using sea snake, sea krait, crocodile, marine turtle, freshwater turtle and monitor lizard *16S* rDNA reference sequences (Table 1). Target length of the amplified fragments ranged from 212 bp (sea snake and sea krait) to 275 bp (crocodile). There were no mismatches with the primers, except for the monitor lizard reference sequences (*Varanus indicus* and *V. panoptes*), which both exhibited one mismatch towards the 5′ end of the forward primer. It is unlikely that this hindered potential amplification of monitor lizards with the *16S* Reptile assay, but may affect efficacy of detection when other (matching) reptile templates are more abundant.

The average pairwise percentage identity for sea snakes (Table 1) across the target region was 94.2% (across 30 single-nucleotide polymorphisms, SNPs); the pairwise percentage identity for congeneric sea snakes averaged 96.4% (min. 93.4%, max. 100%), providing enough variation to distinguish closely related taxa. Across the two congeneric crocodiles and two congeneric monitor lizards (Table 1), the pairwise percentage identity was 95.3% (across 13 SNPs) and 88.8% (across 25 SNPs) respectively. For marine turtles (superfamily: Chelonioidea), the average pairwise percentage identity was 90.9% (across 48 SNPs), and for freshwater turtles (family: Chelidae and Carettochelyidae) it was 74.0% (across 127 SNPs).

In vitro *primer testing*

The *16S* Reptile assay was first tested *in vitro* using tissue extractions (1/10 dilution) of salt water crocodile and flatback turtle. These successfully amplified with an average cycle threshold (C_T) value of 27.1 and 21.9 respectively. The respective extracts matched to NCBI reference sequences of saltwater crocodile and flatback turtle with percentage identities of 100 and 99.6%.

Primers were then tested on the 46 water samples collected at Roebuck Bay and Cooktown. The majority of the eDNA extracts amplified with the *16S* Reptile assay, albeit with high C_T values ranging from 28 to 39, reflecting low template copy numbers. The *16S* Reptile assay yielded a total of 4 037 282 sequencing reads; the mean number of filtered sequences (post-quality, denoising and chimera filtering) was 17 796 ± 25 010 per sample (Table S2 of the Supplementary material). This resulted in a total of 96 taxa being detected with the *16S* Reptile assay (Tables S3, S4 of the Supplementary material), with the majority of taxa being non-reptile (Fig. 2). The highest average proportion of sequencing reads were attributed to bony fish (class: Actinopterygii, 36.7%), followed by amphibians (class: Amphibia, 33.6%), bivalve molluscs (class: Bivalvia, 12.9%), reptiles (class: Reptilia, 8.1%) and mammals (class: Mammalia, 8.1%). Two species of marine turtle (*N. depressus* and *Chelonia mydas*) were detected at 6 of 12 Roebuck Bay sites, and seven

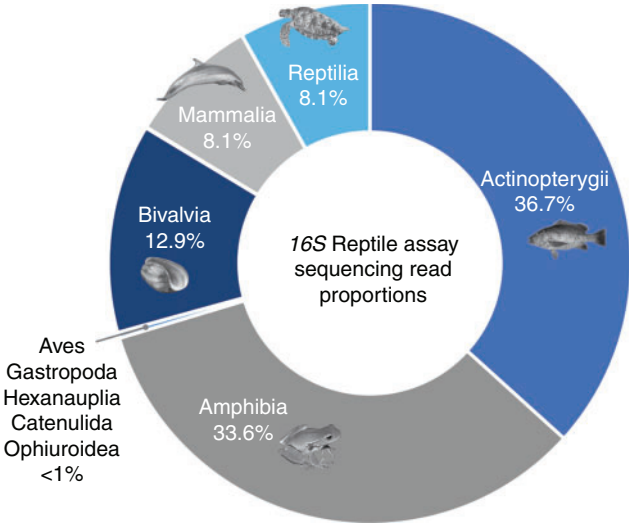

Fig. 2. Proportion of Reptile *16S* sequencing reads attributed at a class level from the environmental DNA (eDNA) study at Roebuck Bay, Western Australia, and Cooktown, Queensland.

largely freshwater-associated reptile taxa were detected at all four Cooktown creek sites (Table 2). The latter included freshwater turtles (*Myuchelys latisternum* and *Emydura*), aquatic and terrestrial snakes (Homalopsidae and *Dendrelaphis calligaster*) and skinks (*Saproscincus basiliscus*, *Carlia longipes* and *Carlia storri*).

Average read depth of the detected reptiles varied from 16 reads (0.5% of average total reads) to 3247 reads (24.2% of average total reads). The percentage identity match of the assigned reptile taxa ranged from 94.6 to 100% (Table 2). The majority of the species assignments had a high percentage identity match (>98% with 100% query coverage). Only the northern tree snake (*D. calligaster*) had a lower percentage identity match of 95.8% (with 100% query coverage). However, the queried eDNA sequence provided no other hits above 90%. This indicates that the eDNA sequence detected may represent intraspecific differentiation from the NCBI reference sequence of *D. calligaster*, or a closely related taxon that is yet to be barcoded and shared on a publicly accessible database. For both the genus and family assignments of *Emydura* and Homalopsidae respectively, there was not enough differentiation (percentage identity difference of ≤1%) between closely related taxa to confidently assign at a species level. However, for both queried eDNA sequences there were no percentage identity matches above 98%, indicating that the detected sequences potentially represent taxa that are yet to be barcoded.

Discussion

The *in vitro* eDNA testing of our *16S* Reptile metabarcoding assay successfully detected two marine turtle species at 6 of 12 Roebuck Bay sites, where they were visually observed in the area on water sampling (Table S1). Flatback turtle (*Natator depressus*) was detected widely across ocean, creek and intertidal sites at Roebuck Bay, whereas green turtle (*Chelonia mydas*) was detected only at Site 4 (ocean), despite being observed at creek and intertidal sites within Roebuck Bay. The principal detection of marine reptiles at Roebuck Bay was not

Table 2. Reptile taxa detected from environmental DNA (eDNA) samples collected at Roebuck Bay (RB), Western Australia, and Cooktown (CK), Queensland

Common name	Scientific name	Distribution	Site(s) detected	Average read depth	Average percentage of total reads	Average percentage identity
Indo-Australian water snakes	Homalopsidae	Northern Australia and Southeast Asia. Widely located in both marine and freshwater, in addition to terrestrial environments.	CK 1	3247	24.2	94.6–94.8
Northern tree snake	*Dendrelaphis calligaster*	Northern Australia, Indonesia, Papua New Guinea and Solomon Islands. Located in terrestrial environments with dense vegetation.	CK 3	92	1	95.8
Pale-lipped shadeskink	*Saproscincus basiliscus*	Queensland, Australia. Located in rainforest environments.	CK 2	500	2.9	98.2
Closed-litter rainbow-skink	*Carlia longipes*	Northern Queensland, Australia and southern Papua New Guinea. Located in open forest and rainforest environments.	CK 1	326	1.6	99.5
Brown bicarinate rainbow-skink	*Carlia storri*	Located in Queensland and south-western Papua New Guinea. Located in the supralittoral zone, shrubland, savanna and forest environments.	CK 1	1773	9.1	98.1–98.6
Flatback turtle	*Natator depressus*	Australian continental shelf. Located in soft-substrate and seagrass environments. Listed as 'Vulnerable' by the *Australian EPBC Act* 1999 and 'Data Deficient' by the IUCN.	RB 1, 3, 4, 5, 9, 11	198	11.6	99.6
Green turtle	*Chelonia mydas*	Indo-Pacific and Atlantic. Located in hard and soft substrate, pelagic and seagrass environments. Listed as 'Vulnerable' by the *Australian EPBC Act* 1999 and 'Endangered' by the IUCN.	RB 4	16	0.5	100
Saw-shelled turtle	*Myuchelys latisternum*	Northern and eastern Australia. Located in freshwater environments such as creeks, rivers, dams and lakes.	CK 1, 3, 4	166	1.6	100
Australian short-necked turtles	*Emydura*	Northern and eastern Australia and in Papua New Guinea. Located in freshwater environments such as creeks, swamps, rivers, dams and lakes.	CK 4	126	2.6	97.2

unexpected, given that the surveyed sites were predominately marine based, with the exception of two estuarine sites. In comparison, the Cooktown sites, which were located solely within estuaries, provided a greater detection range of freshwater and also terrestrial reptile species. Two freshwater turtles (the saw-shelled turtle, *M. latisternum*, and an Australian short-necked turtle, genus: *Emydura*) were detected at three of the Cooktown sites; these eDNA detections coincide within their known distribution ranges across northern and eastern Australia. Terrestrial skinks and a northern tree snake were additionally detected at the Cooktown sites, which is likely to reflect DNA shed into the sampled coastal creeks from drinking, skin shedding or other activities.

A notable detection at Cooktown was that of an Indo-Australian water snake (Homalopsidae). The family Homalopsidae comprises over 50 aquatic and semi-aquatic species that typically inhabit mangrove forests, tropical tidal wetlands and coastal waters from Southeast Asia to northern Australia (Alfaro *et al.* 2008). However, the detected Homalopsidae eDNA sequence could not be resolved to a species or even genus level, because there were no high percentage matches (>98%), typically required for a species assignment, and of the remaining Homalopsidae hits (>90%), there was not enough resolution to confidently distinguish taxa even at the genus level. This indicates that the detected Homalopsidae eDNA sequence at Cooktown represents an Indo-Australian water snake that has not yet been barcoded for the *16S* region and is potentially an undescribed species. Targeted herpetological surveying at this site is recommended to resolve this Homalopsidae eDNA detection.

However, a discrepancy in the performance of the *16S* Reptile assay is that it did not detect any sea snake (Elapidae: Hydrophiinae) species, despite visual observations at 5 of the 12 Roebuck Bay sites (Table S1). Additionally, it failed to detect any saltwater crocodile, despite previous observations in both the Roebuck Bay and Cooktown areas. This lack of detection could be attributed to the non-specificity of the assay or a low shedding rate of reptiles in the environment. Despite the fact that the assay was designed to preferentially amplify reptile taxa, reptiles accounted only for 8.1% of the average proportion of sequencing reads per sample; however, this varied greatly among samples. The detection of the Indo-Australian water snake (Homalopsidae), for example, accounted for 24.2% of the total sequencing reads from Cooktown Site 1. Therefore, even though the *16S* Reptile assay does detect other taxonomic groups (bony fish, amphibians, bivalve molluscs and mammals), it is not exhibiting consistent preferential amplification of these groups above reptiles.

Further optimisation of this assay to reduce non-target amplification is ideal, although this may be complicated given that the primers are located in a highly conserved region of the *16S* rRNA gene. Furthermore, the inclusion of mismatches increases the risk

that rare reptile variants will be excluded from amplification; additionally, the placement of mismatches on 3′ ends may compromise the efficiency of qPCR assays (Wilcox *et al.* 2014). Alternatively, the development of discrete assays for each major reptilian order (i.e. turtles, crocodiles, tuatara, and squamates (lizards and snakes)) may be more effective in refining specificity, but retaining broad-taxonomic amplification. Another possibility is the use of blocking primers, which preferentially binds and restricts amplification of a targeted taxonomic group. Blocking primers have been successfully used in conjunction with metabarcoding assays to increase the specificity of amphibian and bony fish amplicons by reducing the amplification of human DNA (Valentini *et al.* 2016; Sasso *et al.* 2017).

The shedding rate of reptiles may also undermine our ability to detect them by eDNA methods. It has recently been suggested that reptiles may have a relatively lower shedding rate because of their keratinised scales and reduced urine production, and, subsequently, are less detectable than are other mucus-shedding organisms, such as fish and amphibians (Raemy and Ursenbacher 2018; Adams *et al.* 2019). Although this is yet to be explicitly tested, it may explain the inconsistent amplification of reptiles in aquatic environments across multiple studies. For example, giant garter snake (*Thamnophis gigas*) mesocosm experiments reported positive eDNA detection in tanks with snake skin and snake faeces, but no detection with live snakes in tanks, nor at field locations, despite capture of the species with traps (Halstead *et al.* 2017). The collection of water within 1 m of eastern massasauga rattlesnakes (*Sistrurus catenatus*) produced a positive eDNA detection only for 2 of 100 water samples (Baker *et al.* 2020). Conversely, Lacoursière-Roussel *et al.* (2016) successfully used metabarcoding to detect redbelly snake (*Storeria occipitomaculata*), northern watersnake (*Nerodia sipedon*), milksnake (*Lampropeltis triangulum*), snapping turtle (*Chelydra serpentine*) and wood turtle (*Glyptemys insculpta*) in rivers and lakes in Canada; however, the wood turtle was not detected in four rivers that produced positive detections by species-specific qPCR and visual surveying. Overall, there has been a lot more success in the detection of wild turtles than there has been of snakes with eDNA, primarily with species-specific assays (Kelly *et al.* 2014; de Souza *et al.* 2016; Feist *et al.* 2018), with a push to quantify turtle abundance and biomass using eDNA (Adams *et al.* 2019). In regard to crocodiles, only one published study has attempted to amplify crocodile eDNA from the field. However, despite observing West African crocodile (*Crocodylus suchus*) and the Nile monitor (*Varanus niloticus*) in the water at their field sites in Mauritania, they were unable to amplify any crocodile or other reptilian eDNA using a metabarcoding approach (Egeter *et al.* 2018). A low shedding rate may, therefore, limit eDNA detection, despite a well-designed assay that is capable of amplifying the targeted taxa. It is possible that changing the eDNA substrate or method may help improve detections by enriching fractions for target taxa; for example, a plankton-tow might assist in retrieving reptile eDNA (Koziol *et al.* 2019). Nonetheless, an increase in sampling density may be the most feasible approach to increase eDNA sensitivity for aquatic reptiles.

Another limitation to the implementation of eDNA metabarcoding for broad-reptile surveying is potential reference database gaps for DD taxa. At present, only 27.6% of reptile species have been barcoded for the mitochondrial cytochrome *c* oxidase I (*COI*) gene; a short, standardised gene region that has historically formed the primary barcode sequence for animal species (Ratnasingham and Hebert 2007). Only four sea snake species (Elapidae: Hydrophiinae) have been barcoded for this region, and only two of which are distributed in northern Australia. Our assay design targeted the mitochondrial *16S* rDNA gene region, given the development of a *16S* Indo-Pacific sea snake database with a high representation of northern Australian sea snakes (Sanders *et al.* 2013). The development and implementation of eDNA metabarcoding for reptile species, particularly for DD taxa, should ideally be tailored to available reference sequences for the targeted taxonomic group. As such, the development of discrete taxonomic assays, as discussed previously for refining specificity, may be a superior approach to ensure high-resolution assignments (i.e. to a species level). Ultimately, however, it will be easier to implement broad metabarcoding assays with a standardised barcode region, a complete suite of barcoded gene regions, or, ideally, a complete mitochondrial genome for each representative species.

The inconsistent amplification and detection of aquatic reptilian eDNA, despite positive visual and trapping detections at survey sites, indicates that, at present, eDNA methodology provides an unreliable estimate of diversity and community composition among sites. We recommend that aquatic and semi-aquatic reptile shedding rates into various substrates (e.g. water, sediment, soil) are substantially tested before eDNA approaches, in particular metabarcoding, are further applied as reptile survey tools. This will provide greater insight into inconsistencies in amplification among taxonomic groups and whether assays need to be tailored to accommodate this, i.e. the use of species-specific assays for taxa with low shedding rates. An alternative approach to detecting reptiles with potentially low shedding rates would be to explore sampling volumes and subsequent filtering methods, i.e. increasing our standard water replicate volumes from 1 L up to 50 L.

Here, we present a *16S* rDNA primer assay for the broad detection of aquatic and semi-aquatic reptile species in northern Australia. However, constraints around suitable primer-binding regions that can simultaneously amplify deeply diverged reptile lineages has resulted in non-target amplification of other closely related metazoan groups, such as amphibians. If a higher level of specificity is desired, we further recommend that reptile eDNA metabarcoding assays are developed at an order level or lower, and consider the coverage of reference databases for various gene regions. Taken together, we advocate that this *16S* Reptile assay is a valuable addition to the metabarcoding assay 'toolkit' and, like many of the other assays developed (Miya *et al.* 2015; Elbrecht and Leese 2017; Taberlet *et al.* 2018; Nester *et al.* 2020), will be useful when designing or screening environmental samples for reptiles and other taxa.

Conflicts of interest

The authors declare that they have no conflicts of interest.

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