eDNA Metabarcoding Preliminary Report: Blind Cave Eel survey in the Pilbara WA

Prepared for

Rio Tinto
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Prepared by

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Curtin University

21st March 2018

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Signature: 

(Dr Nicole White) **Date: 21st March 2018**

Executive Summary:

Overall Project objective:

The aim of this pilot project was to use an environmental DNA (eDNA) metabarcoding approach to determine presence/absence of Blind Cave Eel (*Ophisternon candidum*) from within and outside the Mesa H project area in the Pilbara region of Western Australia. The data was to be incorporated into an environmental impact statement (EIS) by Rio Tinto. This interim report documents the results to date and will be superseded by the final report on the project which involves replication of data and design of new assays for eel detection.

Outputs:

- Two collection trips in 2017 were conducted (i) June 2017: 91 water samples from 18 bores were collected and filtered by Michelle Guzik (University of Adelaide. (ii) December 2017: 85 water samples were collected and filtered by Jason Alexander (Biota) from 16 bores and one open surface water sample from the Robe River (5x 1 liter replicates). The TrEnD lab at Curtin University successfully isolated DNA from 122 of these samples.
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- As a preliminary first-pass to determine the presence of Blind Cave Eel (*O. candidum*) from 122 water membranes the TrEnD labs Fish 16S assay was used. Although this DNA assay (Fish 16S) isn't specific for Blind Cave Eel our previous research has demonstrated successful amplification of eel DNA.

- The June 2017 water membranes detected the possibility of Blind Cave Eel (*O. candidum*) from bore JW021. The December 2017 water membranes detected the possibility of Blind Cave Eel (*Ophisternon candidum*) from bore 25 and bore RR1. This result is preliminary in nature – detections are currently un-replicated. Additional sites could yield eel signatures as assays are repeated and modified.

- Blind Cave Eel (*O.candidum*) tissue was sourced from the Western Australia Museum (WAM) for comparative purposes. We have sequenced the 16S barcode from this sample.

- Comparisons of the potential Blind Cave Eel (*O. candidum*) DNA sequences from bore JW021 with the National Centre for Biotechnology Information (NCBI) database showed an 85.9% match to *O. bengalense*, an eel-like fish found in northern Australia and 85.9% match to *Synbranchid* sp., a freshwater eel-like fish.

- Comparisons of the potential Blind Cave Eel (*O. candidum*) DNA sequences from bore 25 and RR1 with the NCBI database showed an 87.7%% match to *O. bengalense* and 87.1% match to *Synbranchid* sp.

- Comparisons of the potential Blind Cave Eel (*O. candidum*) DNA sequences from bore JW021, 25 and RR1 with the reference sample obtained from the WAM showed 100% match to the DNA sequences obtained from bore JW021 and 97% match to the DNA sequences from bore 25 and RR1. The genetic sequences in bores 25 and RR1 are 100% similar to each other but distinctly different from the WAM reference or the JW021 sequence. The significance of this genetic difference is unknown.

Recommendations:

- An eDNA approach using a Fish 16S assay has successfully detected Blind Cave Eel from three bores within and outside the Mesa H project area. The sensitivity and reproducibility of these findings still need to be confirmed by the ongoing research program. The successful detection of Blind eel from a non-invasive sample speaks to the promise of eDNA approaches for the detection of taxa in subterranean environments.

1. Introduction

eDNA refers to all genetic material that is recovered from environmental substrates (such as water or sediment). DNA directly from an organism (e.g. microorganisms) as well as trace amounts of DNA that are shed by all organisms is collectively termed eDNA. This preserved, but often degraded, genetic material provides a means to audit species composition and communities at a given location. When combined with recently developed DNA sequencing technologies (termed 'next generation' or 'high-throughput' sequencing), eDNA can provide a wealth of information for studies of introduced marine pests (IMP), biodiversity surveys, food web dynamics, and diet analysis.

Potential applications for monitoring using eDNA include:

- Biosecurity: to detect the presence/absence of introduced species
- Environmental Impact Assessment: baseline/ongoing monitoring and assessment; audit
- Conservation: to detect the presence/absence of threatened species

Rio Tinto has partnered with the Trace and Environmental DNA (TrEnD) Laboratory at Curtin University to develop eDNA tools for Blind Cave Eel (*Ophisternon candidum*) as a means to use DNA-based bio surveys in groundwater monitoring and management.

eDNA sampling has benefits over other traditional survey methods including:

- highly cost-effective monitoring compared to traditional survey methods
- rapid sample collection that is non destructive to habitats, flora and fauna
- non-reliance on individuals with taxonomic expertise, provision of consistent results between monitoring programs, independent of surveyors

The objectives of this project, albeit preliminary, were to use an environmental DNA (eDNA) metabarcoding approach to determine the presence/absence of Blind Cave Eel (*Ophisternon candidum*) a threatened species within the groundwater systems of the Pilbara in Western Australia, specifically the Mesa H area (Rio Tinto). The data was to be incorporated into an environmental impact statement.

2. Methods and Samples

Water samples were collected by Michelle Guzik (University of Adelaide; June 2017) and Jason Alexander (Biota; December 2017) and filtered using a Sentino pump (Pall corporation) as outlined in TrEnD standard operating procedures (available upon request). Turbidity in the water resulted in varying volumes of water that could be filtered between the bores and replicates (see table 1 below and attached electronic data file).

Table 1

Bore water collected by Michelle Guzik (June 2017)								
AWFS ID	**Bore Hole**	**Collection me**	**Date filtered**	**Sample 1 (A)**	**Sample 2 (B)**	**Sample 3 ©**	**Sample 4 (D)**	**Sample 5 (E)**
AWFS-I17-106 (A, B, C, D, E)	JIMWE003	Bailer	13/06/2017	250mL	750mL	1L	1L	650mL
AWFS-I17-107 (A, B, C, D, E)	JW021	Bailer	13/06/2017	700mL	700mL	600mL	600mL	800mL
AWFS-I17-108 (A, B, C, D, E)	BC186	Bailer	13/06/2017	1L	1L	1L	1L	1L
AWFS-I17-109 (A, B, C, D, E)	JW024	Bailer	13/06/2017	1L	1L	1L	700mL	1L
AWFS-I17-110 (A, B, C, D, E)	BC276	Bailer	14/06/2017	1L	500mL	500mL	500mL	250mL
AWFS-I17-111 (A, B, C, D, E)	BC667	Bailer	14/06/2017	250mL	250mL	250mL	1L	1L
AWFS-I17-112 (A, B, C, D, E)	BCP001	Bailer	14/06/2017	250mL (colle	250mL (colle	250mL	1L	1L
AWFS-I17-113 (A, B, C, D, E)	BC458	Bailer	15/06/2017	1L	1L	1L	1L	1L
AWFS-I17-114	Robe River	Container	14/06/2017	700mL	NA	NA	NA	NA
AWFS-I17-115 (A, B, C, D, E)	BUNWO1108	Bailer	15/05/2017	700mL	1L	1L	1L	500mL
AWFS-I17-116 (A, B, C, D)	BUNWB13	Bailer	15/05/2017	1L	750mL	750mL	750mL	NA
AWFS-I17-117 (A, B, C, D, E)	BUNWB09	Bailer	15/06/2017	1L	800mL	800mL	800mL	1L
AWFS-I17-118 (A, B, C, D, E)	BC401	Bailer	16/06/2017	1L	600mL	500mL (colle	1L	1L
AWFS-I17-119 (A, B, C, D, E)	BUNWB08	Bailer	16/06/2017	1L	1L	1L	1L	1L
AWFS-I17-120 (A, B, C, D, E)	BC225	Bailer	15/06/2017	1L	1L	1L	1L	1L
AWFS-I17-121 (A, B, C, D, E)	JIMWE004	Bailer	16/06/2017	1L	1L	1L	800mL	800mL
AWFS-I17-122 (A, B, C, D, E)	BC292	Bailer	16/06/2017	250mL	1L	1L	1L	1L
AWFS-I17-123 (A, B, C, D, E)	BUNWO0773	Bailer	16/06/2017	500mL	500mL	500mL	1L	1L
AWFS-I17-124 (A, B, C, D, E)	PZ11BUN008	Bailer	16/06/2017	600mL	250mL	250mL	250mL	250mL

Bore water collected by Jason Alexander (Biota; December 2017)							
AWFS ID	**Site**	**Easting**	**Northing**	**Date**	**Hole depth**	**Drilled**	**Notes**
AWFS-I18-001 (A,B,C,D,E)	25	432152	7602229	2017-12-13			
AWFS-I18-002 (A,B,C,D,E)	31	432929	7603179	2017-12-13			
AWFS-I18-003 (A,B,C,D,E)	200/Dave Bore	392512.032	7607435.55	2017-12-12			PSS089
AWFS-I18-004 (A,B,C,D,E)	BC186	429578	7587212	2017-12-13			Confirmed eel record 200
AWFS-I18-005 (A,B,C,D,E)	BC401	443563	7574126	2017-12-13			
AWFS-I18-006 (A,B,C,D,E)	Budgie Bore	382494.738	7615587.3	2017-12-12			
AWFS-I18-007 (A,B,C,D,E)	JW023	426138	7590140	2017-12-13			Confirmed Eel record 201
AWFS-I18-008 (A,B,C,D,E)	JW024	427126	7590154	2017-12-13			Confirmed Eel record 201
AWFS-I18-009 (A,B,C,D,E)	MB16MEC0009	393590.062	7601144.51	2017-12-12	43	Drilled 2016	
AWFS-I18-010 (A,B,C,D,E)	MB17MEH0008	417119.5	7600417	2017-12-14	18	29-Aug-17	
AWFS-I18-011 (A,B,C,D,E)	MB17MEH0010	416440	7599848	2017-12-12	30	31-Aug-17	
AWFS-I18-012 (A,B,C,D,E)	MB17MEH0015	416041	7597690	2017-12-14	37	3-Nov-17	
AWFS-I18-013 (A,B,C,D,E)	RR1	419176	7597904	2017-12-14	17.2		depth to water 1.87
AWFS-I18-014 (A,B,C,D,E)	MB16MEC0008	393565	7600580	2017-12-12	36	Drilled 2016	
AWFS-I18-015 (A,B,C,D,E)	MB17MEH0007	417663.8	7600421	2017-12-14	30	29-Aug-17	
AWFS-I18-016 (A,B,C,D,E)	MB17MEH0009	416435.8	7599848	2017-12-12	43	30-Aug-17	
AWFS-I18-017 (A,B,C,D,E)	Control	424478	7597147	2017-12-11			

eDNA was extracted using Qiagen Blood & Tissue DNeasy kits in the TrEnD laboratory at Curtin University in dedicated facilities (inclusive of laboratory clean rooms) suited for eDNA workflows. eDNA derived from the Fish 16S assay was amplified using PCR, followed by sequencing on an Illumina MiSeq in the TrEnD Laboratory. Negative laboratory controls were included (and sequenced if identified as positive) to control for environmental laboratory contamination.

The 16S DNA sequences recovered were compared to the National Centre for Biotechnology Information (NCBI) database for taxonomic identification and to reference Blind Cave Eel (*Ophisternon candidum*) obtained from the WAM (P.34817-001).

3. Results and Discussion

The following table outlines the taxonomic assignments from each bore water sample for which suspected Blind Cave Eel (*Ophisternon candidum*) was amplified and sequenced. Taxonomic assignments are to genera and species with the per cent match genetic similarity to the DNA sequences contained on the reference database (NCBI). At the time of this report, the taxonomic assignments for the DNA sequences obtained from the bore water samples (i.e. DNA 'barcodes') are listed in the table below and the electronic file accompanying this report. The environmental laboratory controls for this work were identified as negative and not included in the Table.

Table 2

Taxonomic Assignments			
AWFS ID	**Bore**	**% match (NCBI)**	**Species**
AWFS-I17-107B	JW021	85.9	Synbranchid sp. VietNam-TC-2002 haplotype 1
		85.9	Ophisternon bengalense voucher MRS haplotype OB1
AWFS-I18-001A	25	87.1	Synbranchid sp. VietNam-TC-2002 haplotype 1
		87.1	Ophisternon bengalense voucher MRS haplotype OB1
AWFS-I18-013A	RR1	87.1	Synbranchid sp. VietNam-TC-2002 haplotype 1
		87.1	Ophisternon bengalense voucher MRS haplotype OB1
AWFS-I18-013C	RR1	87.1	Synbranchid sp. VietNam-TC-2002 haplotype 1
		87.1	Ophisternon bengalense voucher MRS haplotype OB1
AWFS-F18-005 BlindEel_WAM P.34817-001		85.9	Synbranchid sp. VietNam-TC-2002 haplotype 1
		85.9	Ophisternon bengalense voucher MRS haplotype OB1

Fish DNA was detected in a number of sampling locations. This preliminary report is only reporting on, and presenting data from, those bores that yielded eel DNA.

Comparisons of the potential Blind Cave Eel (*O. candidum*) DNA sequences from bore JW021 with the NCBI database showed an 85.9% match to *O. bengalense*, an eel-like fish found in northern Australia and 85.9% match to *Synbranchid* sp., a freshwater eel-like fish (see Table 2). The DNA barcode recovered from JW021 was a 100% match to the reference DNA sequence from the WAM Blind Cave Eel (*O. candidum*) sample. Eel DNA was recovered in one of two replicates from bore JW021

Comparisons of the potential Blind Cave Eel (*O. candidum*) DNA sequences from bore 25 and RR1 with the NCBI database showed an 87.7%% match to *O. bengalense* and 87.1% match to Synbranchid sp. (see table 2). The DNA barcode recovered from bore RR1 and bore 25 was a 97% match to the reference DNA sequence from the WAM Blind Cave Eel (*O. candidum*) sample. Eel DNA was recovered in two of the five replicates from bore RR1 and one of five replicates from bore 25.

The genetic sequences in bores 25 and RR1 are 100% similar to each other but distinctly different from the WAM reference or the JW021 sequence. The significance of this genetic difference is

unknown it may represent variation within the species or a distinct lineage. There is a distinctive DNA 'insertion' in the sequences obtained from JW021 and the WAM reference that is the major distinguishing feature (see Figure 1)

Figure 1: 16S DNA sequences from bore JW021, RR1, 25 and *O. candidum* (WAM P.34817-001)

4. Findings, Recommendations and Concluding remarks

The Fish 16S metabarcoding assay utilised on the bore water samples collected in June and December 2017 detected the potential Blind Cave Eel (*O. candidum*) in three of 34 bores. To our knowledge this represents the first time an eDNA based approach has been used in the ground water system(s) in the Pilbara for the detection of a restricted species. The gaps in the reference databases often prevent definitive assignments to genus and/or species at the time of this report. Although comparisons of the suspected Blind Cave Eel (*O. candidum*) DNA sequences with the DNA sequences obtained from a tissue sample of Blind Cave Eel (*O. candidum*) from the WAM indicate the strong likelihood of Blind Cave Eel (*O. candidum*). Current laboratory work is underway to sequence the complete mitochondrial genome from the WAM reference sample and develop a metabarcoding assay specific for Blind Cave Eel (*O. candidum*). Once completed, the Blind Cave Eel (*Ophisternon candidum*) metabarcoding assay will be used to re-screen the 122 water samples to validate the findings from the Fish 16S assay and potentially find additional bores that may contain Blind Cave Eel (*O. candidum*).

The DNA yields recovered from the bores was low and, as expected, not every replicate from positive wells yielded a positive detection. The sensitivity of the assays and the level of replication appropriate is yet to be determined for bores in the Pilbara. The development of additional assays and optimisation of existing assays (and sampling protocols) is ongoing and will likely enhance the likelihood of detections.

5. Appendices and additional information

- An electronic file titled; Appendix_BlindCaveEel_March2018.xlsx is attached to this report.

- Additional information on sampling and methods are available from TrEnD upon request however the exact nature of the assays and workflow are proprietary.

- Below is a list of acronyms, abbreviations and definitions that may apply to aspects of this report.

% value in data	Represents the percentage similarity of a DNA sequence recovered from a sample compared to reference sequences in a database (e.g. compared to DNA databases such as GenBank or references generated in-house)
(x) value in data	Represents the frequency the % value was recorded in the dataset
16S rRNA	The 16S rRNA refers to a conserved gene region of mitochondrial DNA, which codes for a subunit of the ribosome. 16S rRNA is found in all eukaryotes making it a good candidate for DNA barcoding and is used extensively to detect vertebrate taxa such as fish and mammals.
18S rRNA	The 18S rRNA refers to a conserved gene region of nuclear DNA, which codes for a subunit of the ribosome. 18S rRNA is found in all eukaryotes making it a good candidate for DNA barcoding
18S IMS reference database	Reference 18S rRNA sequences of invasive marine species that are available in DNA databases
Assay	In the context of metabarcoding an assay is a molecular test (using PCR) that is implemented to target a group of taxa within a mixed biological substrate. It is akin to using a magnet to selective enrich for needles (the target) in the context of a haystack (the total DNA from a sample).
COI	The gene region that is being used as the standard barcode for almost all animal groups is a 648 base-pair region of the mitochondrial cytochrome c oxidase 1 gene ("CO1"). COI is proving highly effective in identifying birds, butterflies, fish, flies and many other animal groups. COI is not an effective barcode region in plants because it evolves too slowly, but two gene regions in the chloroplast, matK and rbcL, have been approved as the barcode regions for plants
CO1 IMS reference database	Reference COI sequences of invasive marine species that are available in DNA databases
DNA	Deoxyribonucleic Acid (DNA) is the hereditary material that contains the genetic information of an organism
DNA metabarcoding	Is a genetic technique that simultaneously amplifies and sequences barcode regions

	(e.g. COI, 18S, 16S) of many different species in parallel
eDNA	Environmental DNA (eDNA) refers to genetic material that is recovered from an environmental substrate (e.g. water, sediment, air)
eukaryotes	An organism where cells contain a nucleus surrounded by a membrane and has the DNA bound together by proteins (histones) into chromosomes. The cells of eukaryotes also contain an endoplasmic reticulum and numerous specialised organelles not present in prokaryotes, especially mitochondria, golgi bodies, and lysosomes
Fisheries	Department of Primary Industries and Regional Development, Fisheries Division, Aquatic Biosecurity Section
GenBank	Publically available repository of genetic information. Contains the barcode information of genes that have previously been sequenced
Genome	A genome is all the genetic material of an organism. It consists of DNA (or RNA in RNA viruses). The genome includes both the genes (the coding regions) and the noncoding DNA. In eukaryotes it refers to the genomes of the nucleus, mitochondria and chloroplasts. In prokaryotes, there is a single genome (as they do not contain mitochondria or chloroplasts)
Illumina MiSeq	Next generation sequencing platform developed by the company Illumina
IMP	Introduced marine pests
IMS	Introduced marine species
Metabarcoding assay	A PCR reaction using a specific set of primers that simultaneously amplifies the same gene target from multiple species
Mitochondria	The mitochondrion (plural mitochondria) is a double membrane-bound organelle found in all eukaryotic organisms, although some cells in some organisms may lack them (e.g. Red blood cells). It contains its own genome
Mitogenomes	Refers to the mitochondrial genome
NGS	Next generation sequencing or second generation sequencing refers to massively parallel sequencing technology, as opposed to first generation sequencing or sanger sequencing where only a single template is sequenced at one time
Nucleotide	A compound consisting of a nucleoside linked to a phosphate group. Nucleotides form the basic structural unit of nucleic acids such as DNA
PCR	Polymerase chain reaction (PCR) is the technique that is used to amplify (akin to photocopying DNA) specific regions of the genome from specific groups of taxa
Primer	A short DNA strand (≈20bp in size) used in PCR to target particular groups of organisms and genes. Two of them are required for PCR (a forward and a reverse)
Primer binding site	A primer-binding site is the target region of a genome where the primer attaches to start replication. The primer binding site is on one of the two complementary strands of a double-stranded nucleotide polymer, in the strand which is to be copied, or is within a single-stranded nucleotide polymer sequence
prokaryote	Any of the typically unicellular microorganisms that lack a distinct nucleus and

	membrane-bound organelles and that are classified as a kingdom (Prokaryotae syn. Monera) or into two domains (Bacteria and Archaea)
RNA	Ribonucleic acid (RNA) is a polymeric molecule implicated in various biological roles in coding, decoding, regulation, and expression of genes
rRNA	ribosomal ribonucleic acid is the RNA component of the ribosome, and is essential for protein synthesis in all living organisms
Sequence	DNA sequencing is the process of determining the precise order of nucleotides within a DNA molecule. It includes any method or technology that is used to determine the order of the four bases—adenine, guanine, cytosine, and thymine—in a strand of DNA
Shotgun sequencing	Refers to randomly sequencing short pieces of DNA (≈150bp in size) after shearing or cutting DNA (e.g. fragmenting a genome)
TrEnD	Trace and Environmental DNA laboratory, Curtin University

Follow-up Report: eDNA metabarcoding for Blind Cave Eel survey in the Pilbara WA

Prepared for

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13th August 2018

Report Authors: Dr Nicole White (TrEnD laboratory)

Signature: 

Dr Nicole White **Date:** **13 August 2018**

Executive Summary:

Overall Project objective:

The aim of this pilot project was to use an environmental DNA (eDNA) metabarcoding approach to determine presence/absence of Blind Cave Eel (*Ophisternon candidum*) from within and outside the Mesa H project area in the Pilbara region of Western Australia. The data was to be incorporated into an environmental impact statement (EIS) by Rio Tinto.

Outputs:

- This report is a follow-up of the results from the EelFishCOI 'molecular magnet' designed for Blind Cave Eel (*Ophisternon candidum*) detection. This report presents the eDNA results following on from the report issued to RioTinto on the 13th July 2018 by the TrEnD lab.

- The presence of Blind Cave Eel (*Ophisternon candidum*) DNA was confirmed from two sample sites of the Robe River and bores JW021, JW024, BC186, 25, RR1 and MB17MEH0015. Blind Cave Eel DNA was not previously detected from MB17MEH0015 with the EelFish16S assay reported on 13th July 2018. Re-screening all 122 eDNA water membranes collected in June and December 2017 with Fish16S and EelFishCOI 'molecular magnets' confirms the presence of *O. candidum* from this additional bore.

Recommendations:

- This is the first time eDNA metabarcoding methods have been implemented in the Pilbara groundwater systems. The Fish16S, EelFish16S and EelFishCOI metabarcoding assays designed by the TrEnD lab indicates that an eDNA approach is able to detected Blind Cave Eel (*Ophisternon candidum*) from the survey area as a non-invasive method. Blind Cave Eel (*Ophisternon candidum*) DNA was isolated and DNA sequenced from 6 bores sampled, in addition to two open surface water samples collected from the Robe River, which indicates eDNA metabarcoding is capable of detecting threatened species. Finally it is worth noting that these eDNA assays are performing at the limits of detection and further gains in sensitivity may be achievable with optimised sampling.
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1. Introduction

eDNA refers to all genetic material that is recovered from environmental substrates (such as water or sediment). DNA directly from an organism (e.g. microorganisms) as well as trace amounts of DNA that are shed by all organisms is collectively termed eDNA. This preserved, but often degraded, genetic material provides a means to audit species composition and communities at a given location. When combined with recently developed DNA sequencing technologies (termed 'next generation' or 'high-throughput' sequencing), eDNA can provide a wealth of information for studies of introduced marine pests (IMP), biodiversity surveys, food web dynamics, and diet analysis.

Potential applications for monitoring using eDNA include:

- Biosecurity: to detect the presence/absence of introduced species
- Environmental Impact Assessment: baseline/ongoing monitoring and assessment; audit
- Conservation: to detect the presence/absence of threatened species

Rio Tinto has partnered with the Trace and Environmental DNA (TrEnD) Laboratory at Curtin University to develop eDNA tools for Blind Cave Eel (*Ophisternon candidum*) as a means to use DNA-based bio surveys in groundwater monitoring and management.

eDNA sampling has benefits over other traditional survey methods including:

- highly cost-effective monitoring compared to traditional survey methods
- rapid sample collection that is non destructive to habitats, flora and fauna
- non-reliance on individuals with taxonomic expertise, provision of consistent results between monitoring programs, independent of surveyors

The objectives of this project were to use an environmental DNA (eDNA) metabarcoding approach to determine the presence/absence of Blind Cave Eel (*Ophisternon candidum*) a threatened species within the groundwater systems of the Pilbara in Western Australia, specifically the Mesa H area (Rio Tinto). The data is to be incorporated into an environmental impact statement.

2. Methods and Samples

Please refer to report "**eDNA Metabarcoding Report: Blind Cave Eel survey in the Pilbara WA**" issued to RioTinto by the TrEnD lab on 13th July 2018.

3. Results and Discussion

Table 1 list the water samples from each bore and those for which Blind Cave Eel (*Ophisternon candidum*) were detected with the Fish16S, EelFish16S and EelFishCOI metabarcoding assays. DNA sequences obtained for *O. candidum* can be found in the Supplementary Information accompanying this report.

Table 1: Results of eDNA metabarcoding (Fish16S, EelFish16S, EelFishCOI) from groundwater for Blind Cave Eel (*Ophisternon candidum*) detection.

TrEnD Sample ID	Site (bore code)	Field Trip	Ophisternon candidum DNA detected?
AWFS-I17-106	JIMWE003	Jun-17	No
AWFS-I17-107	JW021	Jun-17	Yes
AWFS-I17-108	BC186	Jun-17	No
AWFS-I17-109	JW024	Jun-17	No
AWFS-I17-110	BC276	Jun-17	No
AWFS-I17-111	BC667	Jun-17	No
AWFS-I17-112	BCP001	Jun-17	No
AWFS-I17-113	BC458	Jun-17	No
AWFS-I17-114	Robe River (RR)	Jun-17	Yes
AWFS-I17-115	BUNWO1108	Jun-17	No
AWFS-I17-116	BUNWB13	Jun-17	No
AWFS-I17-117	BUNWB09	Jun-17	No
AWFS-I17-118	BC401	Jun-17	No
AWFS-I17-119	BUNWB08	Jun-17	No
AWFS-I17-120	BC225	Jun-17	No
AWFS-I17-121	JIMWE004	Jun-17	No
AWFS-I17-122	BC292	Jun-17	No
AWFS-I17-123	BUNWO0773	Jun-17	No
AWFS-I17-124	PZ11BUN008	Jun-17	No
AWFS-I18-001	25	Dec-17	Yes
AWFS-I18-002	31	Dec-17	No
AWFS-I18-003	200/Dave Bore	Dec-17	No
AWFS-I18-004	BC186	Dec-17	Yes
AWFS-I18-005	BC401	Dec-17	No
AWFS-I18-006	Budgie Bore	Dec-17	No
AWFS-I18-007	JW023	Dec-17	No
AWFS-I18-008	JW024	Dec-17	Yes
AWFS-I18-009	MB16MEC0009	Dec-17	No
AWFS-I18-010	MB17MEH0008	Dec-17	No
AWFS-I18-011	MB17MEH0010	Dec-17	No
AWFS-I18-012	MB17MEH0015	Dec-17	Yes
AWFS-I18-013	RR1	Dec-17	Yes

AWFS-I18-014	MB16MEC0008	Dec-17	No
AWFS-I18-015	MB17MEH0007	Dec-17	No
AWFS-I18-016	MB17MEH0009	Dec-17	No
AWFS-I18-017	Control (RR)	Dec-17	Yes

4. Findings, Recommendations and Concluding remarks

At the time of report "**eDNA Metabarcoding Report: Blind Cave Eel survey in the Pilbara WA**" issued to RioTinto by the TrEnD lab on 13th July 2018, the water samples identified as positive from the EelFishCOI and Fish16S metabarcoding assays had not been DNA sequenced and bioinformatically processed.

The Fish16S, EelFish16S and EelFishCOI assays utilised on bore water samples collected in June and December 2017 detected Blind Cave Eel (*Ophisternon candidum*) from two sample locations of the Robe River, in addition to bores JW021, JW024, BC186, 25, RR1 and MB17MEH0015. To our knowledge this represents the first time an eDNA based metabarcoding approach, specific for *Ophisternon candidum*, has been applied to samples from ground water system(s) in the Pilbara, Western Australia. Sequencing the mitochondrial genome of *Ophisternon candidum* (WAM P.34817-001) has provided definitive assignments of DNA sequences from eDNA groundwater samples to genus and species.

5. Appendices and additional information

- An electronic file titled; SupplementaryInformation_EelFish16SandCOI_August2018.xlsx is attached to this report.

- Additional information on sampling and methods are available from TrEnD upon request however the exact nature of the assays and workflow are proprietary.

- Below is a list of acronyms, abbreviations and definitions that may apply to aspects of this report.

% value in data	Represents the percentage similarity of a DNA sequence recovered from a sample compared to reference sequences in a database (e.g. compared to DNA databases such as GenBank or references generated in-house)
(x) value in data	Represents the frequency the % value was recorded in the dataset
18S rRNA	The 18S rRNA refers to a conserved gene region of nuclear DNA, which codes for a subunit of the ribosome. 18S rRNA is found in all eukaryotes making it a good candidate for DNA barcoding
18S IMS reference database	Reference 18S rRNA sequences of invasive marine species that are available in DNA databases
Assay	In the context of metabarcoding an assay is a molecular test (using PCR) that is implemented to target a group of taxa within a mixed biological substrate. It is akin to using a magnet to selective enrich for needles (the target) in the context of a haystack (the total DNA from a sample).
COI	The gene region that is being used as the standard barcode for almost all animal groups is a 648 base-pair region of the mitochondrial cytochrome c oxidase 1 gene ("CO1"). COI is proving highly effective in identifying birds, butterflies, fish, flies and many other animal groups. COI is not an effective barcode region in plants because it evolves too slowly, but two gene regions in the chloroplast, matK and rbcL, have been approved as the barcode regions for plants
CO1 IMS reference database	Reference COI sequences of invasive marine species that are available in DNA databases
DNA	Deoxyribonucleic Acid (DNA) is the hereditary material that contains the genetic information of an organism
DNA metabarcoding	Is a genetic technique that simultaneously amplifies and sequences barcode regions (e.g. COI, 18S, 16S) of many different species in parallel
eDNA	Environmental DNA (eDNA) refers to genetic material that is recovered from an environmental substrate (e.g. water, sediment, air)

eukaryotes	An organism where cells contain a nucleus surrounded by a membrane and has the DNA bound together by proteins (histones) into chromosomes. The cells of eukaryotes also contain an endoplasmic reticulum and numerous specialised organelles not present in prokaryotes, especially mitochondria, golgi bodies, and lysosomes
Fisheries	Department of Primary Industries and Regional Development, Fisheries Division, Aquatic Biosecurity Section
GenBank	Publically available repository of genetic information. Contains the barcode information of genes that have previously been sequenced
Genome	A genome is all the genetic material of an organism. It consists of DNA (or RNA in RNA viruses). The genome includes both the genes (the coding regions) and the noncoding DNA. In eukaryotes it refers to the genomes of the nucleus, mitochondria and chloroplasts. In prokaryotes, there is a single genome (as they do not contain mitochondria or chloroplasts)
Illumina MiSeq	Next generation sequencing platform developed by the company Illumina
IMP	Introduced marine pests
IMS	Introduced marine species
Metabarcoding assay	A PCR reaction using a specific set of primers that simultaneously amplifies the same gene target from multiple species
Mitochondria	The mitochondrion (plural mitochondria) is a double membrane-bound organelle found in all eukaryotic organisms, although some cells in some organisms may lack them (e.g. Red blood cells). It contains its own genome
Mitogenomes	Refers to the mitochondrial genome
NGS	Next generation sequencing or second generation sequencing refers to massively parallel sequencing technology, as opposed to first generation sequencing or sanger sequencing where only a single template is sequenced at one time
Nucleotide	A compound consisting of a nucleoside linked to a phosphate group. Nucleotides form the basic structural unit of nucleic acids such as DNA
PCR	Polymerase chain reaction (PCR) is the technique that is used to amplify (akin to photocopying DNA) specific regions of the genome from specific groups of taxa
Primer	A short DNA strand (≈20bp in size) used in PCR to target particular groups of organisms and genes. Two of them are required for PCR (a forward and a reverse)
Primer binding site	A primer-binding site is the target region of a genome where the primer attaches to start replication. The primer binding site is on one of the two complementary strands of a double-stranded nucleotide polymer, in the strand which is to be copied, or is within a single-stranded nucleotide polymer sequence
prokaryote	Any of the typically unicellular microorganisms that lack a distinct nucleus and membrane-bound organelles and that are classified as a kingdom (Prokaryotae syn. Monera) or into two domains (Bacteria and Archaea)
RNA	Ribonucleic acid (RNA) is a polymeric molecule implicated in various biological roles in coding, decoding, regulation, and expression of genes

rRNA	ribosomal ribonucleic acid is the RNA component of the ribosome, and is essential for protein synthesis in all living organisms
Sequence	DNA sequencing is the process of determining the precise order of nucleotides within a DNA molecule. It includes any method or technology that is used to determine the order of the four bases—adenine, guanine, cytosine, and thymine—in a strand of DNA
Shotgun sequencing	Refers to randomly sequencing short pieces of DNA (≈150bp in size) after shearing or cutting DNA (e.g. fragmenting a genome)
TrEnD	Trace and Environmental DNA laboratory, Curtin University