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Trialling new techniques for assessing terrestrial biodiversity in data-poor environments

Final report

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Abbreviations and acronyms

bp ..... base pair

DNA..... deoxyribonucleic acid

eDNA..... environmental DNA

HTS ..... high throughput sequencing

OUT..... operational taxonomic unit

PCR..... polymerase chain reaction

qPCR..... quantitative PCR

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Summary

The aim of this project was to develop a method for detecting multiple terrestrial species using environmental DNA (eDNA). A suite of PCR primers was optimised using DNA from a tissue library of Northern Territory target animal species and an analysis pipeline was developed. The approach was tested using samples from two study sites and results compared to camera trap data. There was a poor match with camera data and negative control water samples also amplified target DNA but with no particular pattern. To operationalise this approach, the field sampling would need to be repeated with larger volumes of water to improve DNA yield and allow an accurate comparison to camera or count data.

1. Introduction and overview of the field of research

Environmental DNA (eDNA) has become widely adopted as a tool for species detection in biological surveying (Taberlet, Bonin, Zinger, & Coissac, 2018). eDNA is DNA that originated from live organisms and persists for some time in environmental samples. It derives from a mixture of live and dead cells, lysed cells, cellular components and some exposed DNA. It can be collected from a range of substrates such as soil, surfaces of rocks, or filtered from air. However, filtering water and purifying DNA from the biological material captured by fine filters is the most common way to obtain eDNA (Thomsen et al. 2012; Valentini et al. 2016; Taberlet et al. 2018).

There are two main experimental procedures that can be analysed to generate information about the organisms that are represented in a sample of eDNA: Single-species eDNA detection and eDNA metabarcoding, which detects multiple species in parallel. Both approaches depend on the ability of polymerase chain reaction (PCR) technology to copy small numbers of eDNA molecules (Wilcox et al. 2013). The term 'DNA metabarcoding' (Taberlet, Coissac, Pompanon, Brochmann, & Willerslev, 2012) is an extension to multiple species of the term 'DNA barcoding' that was coined earlier to refer to single species identification of animals from DNA sequences (Hebert Paul D. N., Cywinska Alina, Ball Shelley L., & deWaard Jeremy R., 2003). Quantitative PCR (qPCR) technologies are an extension of the PCR technology that allow the PCR amplification reaction to be monitored in real time. The value of qPCR is that it allows the strength of the PCR amplification to be related to the number of target DNA molecules in the DNA extract being studied. qPCR methods or, more recently, the related digital droplet PCR (Doi et al. 2015), are the primary technologies for single-species eDNA studies.

Singe-species eDNA tests have the advantage of being very reliable in detecting a specific target species in the complex mixtures of molecules in eDNA. The tests themselves can also be studied carefully to determine sensitivity and rates of false-negative and false-positive detection. However, single-species tests are expensive to develop. Single-species qPCR tests are particularly valuable if false-positive or false-negative detection will strongly affect the interpretation of a study. There are specific methods for measuring the rate at which these errors occur (Furlan & Gleeson, 2017).

For detection of more than ~4 species at one time, eDNA metabarcoding is the approach of choice (Valentini et al. 2016; Taberlet et al. 2018). eDNA metabarcoding approaches have a different application in that they produce lists of species present in an eDNA sample. These methods all rely on PCR by 'group-specific' PCR primer sets (Jarman, Deagle, & Gales, 2004). Group-specific PCR primers target short DNA regions that are present in most or all members of the target group. The PCR reaction copies all of the DNA present between two of these PCR primer binding sites. The regions between the primer binding sites are called 'DNA metabarcodes' or 'metabarcodes.' The key to eDNA metabarcoding is to design PCR primer sets that bind regions consistent in all target species, but variable in the metabarcode region between the primer binding sites (Jarman et al. 2004). Current methods for analysis after PCR amplification rely on high throughput parallel DNA sequencing technologies, generally called 'next generation sequencing' or 'high throughput sequencing (HTS).'

Metabarcoding approaches are used for studying biodiversity. Biodiversity is classically subcategorised as 'alpha' biodiversity – all the species in one area; 'beta' biodiversity – the differences between two areas; and 'gamma' biodiversity – the total list of species in a region (Whittaker WH, 1960). Metabarcoding is especially good at measuring gamma diversity because it is sensitive and can detect a wider range of species than other biodiversity surveying methods. If applied to multiple samples from a region, it will generally identify more species than any other surveying technology. However, detection rates are biased, and some groups of species will be missed, while others are preferentially detected due to a variety of technical factors. In this sense, metabarcoding is no worse than other biodiversity surveying methods, but because it is new,

researchers in the field have taken care to assess its capacities. The technical biases combined with high sensitivity make metabarcoding also suitable for beta diversity estimation (Bista et al. 2017). Essentially, changes in biodiversity of time or space can be measured, even if there is some bias in recording the exact composition of a community. However, metabarcoding is not suited for measuring alpha diversity. Where the alpha diversity measurement is confounded by the tendency for inflation of number of species because DNA variation does not directly correspond to species differences (Taberlet et al. 2018).

These caveats do not mean the eDNA metabarcoding is a poor tool for biodiversity surveying. In fact, it remains one of the most sensitive and cost-effective approaches for studying a number of ecological questions. The real issue is that eDNA survey results must be interpreted carefully and not taken to be a direct representation of all species in an environment.

Northern Australia has significant advantages as a site for eDNA detection because it has widespread presence of surface water, the key substrate for eDNA purification. The potential difficulties for eDNA studies in this area are that the water will frequently be turbid as a result of high rates of photosynthetic organism growth, or frequent re-suspension of sediment by large animal disturbance, which is a known problem in similar environments (Egeter et al. 2018). Approaches to aquatic eDNA sampling that do not depend on water filtering are likely to be superior (Williams, Huyvaert, & Piaggio, 2016). The other potential difficulty with sampling in this environment is that DNA degradation is heat dependent and to some extent dependent upon bacterial activity. DNA degradation affects detectability of sequences by PCR (Deagle, Eveson, & Jarman, 2006). Northern Australia is hot and much of its water will have high levels of microbial activity, so many practitioners of eDNA research would expect low detection rates for eDNA sampled in this region. However, the single-species detection of Gouldian finch eDNA (Day et al, 2019 and Appendix 1) suggests that even with these confounding factors, eDNA detection by single-species qPCR is feasible.

Another way to consider the high DNA degradation rates likely to be occurring in northern Australia water is that these conditions will result in shorter persistence times for eDNA which is an advantage in measuring occupancy by species of interest. eDNA can persist for long periods under some environmental conditions (Piaggio et al. 2014; Pilliod, Goldberg, Arkle, & Waits, 2014) and this is the basis for the whole field of ancient DNA research (Gilbert, Bandelt, Hofreiter, & Barnes, 2005). The hotter conditions simply reduce the sensitivity of eDNA tests, but in colder environments the sensitivity causes difficulty when residence of a target species can be detected up to a month after organism presence (Balasingham, Walter, & Heath, 2017).

The availability of reference DNA sequences for species identification is a specific issue for northern Australian species because of the paucity of DNA barcoding research focused in this area. However, this situation is similar in most parts of the world. It is intuitively appealing to have DNA sequences identified in eDNA be perfectly matched to DNA sequences unequivocally derived from well-curated specimens of species that have been identified by a taxonomic expert. This is the paradigm that DNA barcoding established (Hebert Paul D. N. et al. 2003). The reality for DNA metabarcoding studies in most cases is quite different. In practise, small proportions of reads in each sample will be unequivocally identifiable following this perfect match concept, even in well-characterised environments. This reality is the result of eDNA metabarcoding being an exquisitely sensitive tool that can detect very small quantities of DNA (Balasingham et al. 2017). It is not uncommon for water samples to contain DNA from several hundred, or even thousands of species (Koziol et al. n.d.; Stat et al. 2017; Berry et al. 2019). Many of these will not have DNA reference database sequences.

This situation has been recognised for a very long time in the field. The whole field of DNA metabarcoding is a direct copy of the earlier field of molecular microbiology, where bacterial DNA sequences were used to study diversity. In this field, especially since species definitions are less clear than for animals (where they are still often not clear) the concept of ‘Operational Taxonomic

Units (OTUs)' was developed. These are DNA-sequence based representatives of a unit of biodiversity approximately equivalent to a species. OTUs have a great deal of value in eDNA metabarcoding work because they allow a species to be detected without giving it a name immediately. If the same OTU shows up in multiple locations and samples, then it is clear that this species is important (Berry et al. 2019). OTUs can then be identified. The first step in this process is to use phylogenetic methods where DNA sequences are compared to each other to infer likely relationships among them. As OTUs are based on DNA sequences, they can be compared to existing databases of similar sequences and even though an exact match will not be present, they can be assigned at a higher taxonomic level. For most DNA metabarcode regions, all families of animals will have a representative, so essentially every OTU can be assigned at family level. In some areas, assignment to genus will be possible. It is frequently then possible to narrow the list of possible species that an OTU represents to only a few possibilities based on known distributions of species (Stat et al. 2017).

Water filtering for eDNA in the field followed by cold storage of filters is the standard protocol for eDNA studies around the world (Valentini et al. 2016). However, many alternative approaches exist, the most common being to sample water and return it to a lab in sterile bottles for processing there. Given that in northern Australia, many samples will be collected in remote areas, alternative approaches to filtering in the field and cold storage of samples should be tested. A recent study suggested that Longmire's solution provides a refrigeration and filtering-free approach for eDNA preservation (Williams et al. 2016). This strategy and other alternatives should be trialled in consultation with community groups, rangers, government representatives and any other people involved in sampling to determine a method that will provide sound scientific results, as well as being feasible for field operators. Once a method has been established, it is important to standardise the sampling protocol. It is advised that an instructional video recording be made to allow any new operators to easily comprehend the procedure, an approach that has worked well in similar situations with multiple field workers (McInnes et al. 2017).

The optimal approach for ensuring species detection by DNA metabarcoding is a two-step PCR-based procedure for creating libraries for high-throughput sequencing. This is essentially the procedure used here (McInnes et al. 2016). The primary PCR reaction is performed with two PCR primers that have an extension sequence and the 'P5' and 'P7' sequence adaptors that are an essential part of the Illumina MiSeq high-throughput DNA sequencing system. The 'tail' of DNA can then be targeted by a secondary PCR with primers that have a 3' end specific to the tail. The second-round primers have short 5–7 bp 'indexes' that can be used to identify specific samples. A different combination of indexes for the forward and reverse primer can be used, so for example, 8 forward primer indexes and 12 reverse primer indexes give a set of 96 combinations (8 × 12 = 96).

DNA sequence data produced by the MiSeq needs to be quality filtered, converted to FASTA files with forward and reverse reads combined into a single sequence. Sequences then need to be sorted by barcodes into separate files corresponding to the individual samples. A range of potential pipelines for analysis follow from this point, with examples provided by (Koziol et al. n.d.; Stat et al. 2017). A variety of tools have been produced in the past two years to improve DNA metabarcoding OTU analysis, such as 'vsearch' for OTU clustering (Rognes, Flouri, Nichols, Quince, & Mahé, 2016); and 'lulu' for post-clustering curation (Frøslev et al. 2017).

eDNA metabarcoding has great potential as a tool for detection of vertebrate and other animal species from aquatic eDNA sampling in northern Australia. Although not perfect, it is currently regarded as a more powerful tool than any known equivalents (Valentini et al. 2016; Taberlet et al. 2018). It was previously thought that eDNA sampling in tropical environments might have a poor rate of success. Several recent studies contradict this assumption and work by Day et al. 2019 on Gouldian finch eDNA detection proves its feasibility *in situ* in the Northern Territory.

2. Approach

We used a multi-step approach to develop a multi-species terrestrial eDNA workflow:

1. prepare a list of target species
2. select primer sequences that are likely to amplify these species
3. compile a reference database for these target species to distinguish those with sequences available in the database from those we need to sequence
4. establish a DNA reference library for northern Australian birds and mammals
5. develop an analysis pipeline and test it against a mock community of target animals
6. operationalise the method with a field study.

3. Results

3.1 Prepare a list of target species

A number of data-poor species of interest were targeted because they have a wide geographical range, are difficult to sample using current systematic techniques and have specific habitat requirements. Introduced predators were also selected. The list is shown in Table 3.1.

Table 3.1. Target species for eDNA detection. Location refers to taxa observed at two key locations important for fauna surveys, Wollogorang Station (W) and the MacDonnell Ranges (M). Category (threatened, difficult to survey or threat) is shown because this was a key decider for which species to include in the target list.

Target species	Common name	Location	Category *1= threatened* *2= difficult to survey* *3= threat*
Zyzomys palatalis	Carpentarian rock-rat	W	1
Zyzomys argurus	Common rock-rat	W	4
Petrogale wilkinsi	Wilkin's rock-wallaby	W	2
Petrogale brachyotis	Short-eared rock-wallaby	W	2
Pseudantechinus bilarni	Sandstone false antechnius	W	1,2
Pseudantechinus mimulus	Carpentarian antechinus	W	2(4)
Planigale maculata	Common planigale	W	2
Petropseudes dahli	Rock ringtail possum	W	2
Amytornis dorotheae	Carpentarian grass-wren	W	1,2
Tachyglossus aculeatus	Echidna	W	2
Trichosurus vulpecula	Common brush-tailed possum	M	1,2
*Zyzomys pedunculatus**	Central rock-rat	M	1
Petrogale lateralis	Black-footed rock-wallaby	M	1,2
Polytelis alexandrae	Princess parrot	M	1,2
Felis catus	Cat	W M	3
Canis lupus	Dingo	W M	4
Sus scrofa	Pig	W	3
Erythrotriorchis radiatus	Red goshawk	M	1,2
Geophaps smithii	Partridge pigeon	M	1,2
Ardeotis australis	Australian bustard	M	2
Burhinus grallarius	Bush-stone curlew	M	2
Simalia oenpelliensis	Oenpelli python	M	1,2
Bellatorias obiri	Arnhem Land egernia	W	2
Lagorchestes conspicillatus	Spectacled hare wallaby	M	2
Hydromys chrysogaster	Water rat	W	2
Dromaius novaehollandiae	Emu	M	2

3.2 Select primer sequences that are likely to amplify these species

The ability of DNA metabarcoding assays to detect a particular species depends on the potential to amplify the metabarcodes via polymerase chain reaction (PCR) (Deagle et al. 2014). To assess the potential for eDNA detection of the target species listed in Table 3.1, ecoPrimers software (Riaz et al. 2011) was used to search the database using previously proven PCR primer sets (Table 3.2). A custom Python script was used to recreate all possible primers that are present when a degenerate PCR primer is used for amplifying DNA metabarcode regions. For example, the PCR primer AACGGACTRCCGATYCCC has two degenerate sites, R (G/A) and Y (C/T) so when this primer is synthesised, it becomes an equal mix of four different primers with the combinations G-C, G-T, A-C, A-T of bases at the degenerate sites. For each previously published primer shown in Table 3.2, each possible primer combination was used. All target species (Table 3.1) are potentially detectable by the PCR primer sets 18Smsq (hereafter referred to as 'Euk'), Leray COI and Vert01. The birds are also detectable by Aves02 and the mammals by Mamm02. During *in silico* testing, one base mismatch was allowed between primer and primer binding site, except in the terminal 5 bases of the primer.

Table 3.2. eDNA metabarcoding PCR primer sets for detection of a wide range of animals, with a specific focus on vertebrates. Each of these primer sets could theoretically amplify a DNA metabarcode from all the species within the target range identified in Table 3.1 above.

Target group	Primer name and DNA region	Forward primer	Reverse primer	Reference
Eukaryota	18Smsq, 18S nuclear rDNA	CACCGCCCGTCGCTACTACCG	GGTTCACCTACGGAAACCTTGTTACG	(McInnes et al. 2016)
Metazoa	Leray COI Mitochondrial COI	GGWACWGGWTGAACWGTWTAYCCYCC	GGRGGRTASACSGTTCASCCSGTSCC	(Leray et al. 2013)
Vertebrates	Vert01 12S mitochondrial rDNA	TTAGATACCCCACTATGC	TAGAACAGGCTCCTCTAG	(Riaz et al. 2011)
Frogs	Batr01 12S mitochondrial rDNA	ACACCGCCCGTCACCCT	GTAYACTTACCATGTTACGACTT	(Valentini et al. 2016)
Birds	Aves02 12S mitochondrial rDNA	GAAAATGTAGCCCATTTCTTCC	CATACCGCCGTCGCCAG	(Taberlet et al. 2018)
Mammals	Mamm02 16S mitochondrial rDNA	CGAGAAGACCCTRTGGAGCT	CCGAGGTCRCCCCAACC	(Taberlet et al. 2018)

3.3 Compile a reference database for target species to identify sequence status

The Aves02, Vert01 and Mamm02 primers (Table 3.2) were tested *in silico* with Geneious™ using the publicly available databases at the National Center for Biotechnology Information (NCBI), the Barcode of Life Data System and the Atlas of Living Australia. Briefly, for each key species, we searched the databases to identify if existing sequences were available for the targeted 12S and 16S rDNA genes (Table 3.3). Approximately 52% of the species in Table 3.3 lacked a reference sequence that matched the selected primers in existing databases, making eDNA detection unlikely for those species. Based on the availability of the sequences and sample specimens, we then assigned a priority ranking for sequencing for the expected animals found at Wollogorang and the arid zone sites in the Northern Territory.

*Table 3.3. Target species for the selected Wollogorang and arid zone study sites. * A blank cell indicates we do not hold reference tissue sample. ** NID = not in database.*

Target species (from Wollogorang, Central and Broad ranges)	Reference tissue available*	No. of gene sequences (any)	Vertebrates: Vert01, 12S mitochondrial rDNA		Birds: Aves02, 12S mitochondrial rDNA		Mammals: Mamm02, 16S mitochondrial rDNA		Sequencing priority (high/med)
			Vert01 12S seq available	Primers will detect this sp.	Aves02 12S seq available	Primers will detect this sp.	Mamm02 16S seq available	Primers will detect this sp.	
Zyzomys palatalis	Yes	1	N	NID	N	NID	N	NID	H
Zyzomys argurus	Yes	4	N	NID	N	NID	N	NID	H
Hydromys chrysogaster		10	N	NID	N	NID	N	NID	H
Petropseudes dahli		0	N	NID**	N	NID	N	NID	H
Trichosurus vulpecula	Yes	19	Y	Y	Y	Y	Y	Y	M
Petrogale wilkinsi		2	N	NID	N	NID	N	NID	H
Petrogale lateralis		72	N	NID	N	NID	N	NID	H
Petrogale brachyotis	Yes	214	Y	Y	Y	Y	Y	Y	M
Lagorchestes conspicillatus	Yes	21	Y	Y	Y	Y	Y	Y	M
Planigale maculata	Yes	61	Y	Y	Y	Y	Y	Y	M
Pseudantechinus mimulus	Yes	8	Y	Y	Y	Y	Y	Y	M
Pseudantechinus bilarni	Yes	9	Y	Y	Y	Y	Y	Y	M
Tachyglossus aculeatus	Yes	4	Y	Y	Y	Y	Y	Y	M
Felis catus	Yes	19	Y	Y	Y	Y	Y	Y	M
Canis lupus	Yes	39	Y	Y	Y	N	Y	Y	M
Sus scrofa		12,285	Y	Y	Y	Y	Y	Y	M
Erythrotriorchis radiatus		1	N	NID	N	NID	N	NID	H
Geophaps smithii		3	N	NID	N	NID	N	NID	H
Amytornis dorotheae		5	N	NID	N	NID	N	NID	H

Ardeotis australis		0	N	NID	N	NID	N	NID	H
Polytelis alexandrae		7	N	NID	Y	Y	Y	Y	H
Dromaius novaehollandiae		40,709	Y	Y	Y	Y	Y	Y	M
Burhinus grallarius	Yes	47	Y	Y	Y	Y	Y	Y	M
Bellatorias obiri		0	N	NID	N	NID	N	NID	H
Simalia oenpelliensis		2	N	NID	N	NID	N	NID	H

3.4 Establish a mock community to test the primers and bioinformatics pipeline

3.4.1 DNA extraction from tissue samples and PCR amplification

We developed a mock-community DNA sequence reference library using locally sourced animal tissue (Table 3.4). This mock community was used to check that the steps from DNA extraction through to eDNA sample taxonomic assignments were accurate. DNA was extracted and purified with a DNeasy Blood and Tissue DNA extraction kit (Qiagen). To test whether the selected primers could amplify the DNA of the mock community, 1 µL of the purified DNA was amplified by PCR with the Aves02, Mamm02 and Vert01 primers from Table 3.2, using PerfeCTa® qPCR ToughMix™ (Quanta Biosciences). Thermal cycling conditions for the primer pairs were 95°C, 10 min; 50 cycles of 95°C 15 s, 64°C 60 s. Amplification products were separated by electrophoresis at 5 $V.cm^{-1}$ on 2% agarose TBE gels. Gel electrophoresis indicated that the Vert01 primers may not amplify all tissues and could be prone to non-specific binding (data not shown). Tested species that failed to amplify with the Vert01 primers were *Smintopsis virginiae*, *Zyzomys woodwardi*, *Pseudantechinus ningbing*, *Gallus gallus* and the *Todiramphus pyrrhopygius*. Additional bands were also detected for the *Mesembriomys macrurus*, *Crocodylus johnsoni* and *Poephila personata*. Repeated PCRs tests using the alternative Euk primers (Table 3.2) resulted in all tissues amplifying without non-specific bands (data not shown). Consequently, the Vert01 primers were replaced by the Euk primers for the rest of the study. The mock community was created by aliquoting 1µL of pure DNA from 15 reference library targets into a single 1.5mL Eppendorf Tube® containing 30µL of 10mM Tris solution. Duplicate samples of the mock community were sequenced to assess the quality of the sequencing and downstream taxonomy assignments.

Table 3.4. Species used to create the mock community for quality control.

Animal ID	DNA conc (ng/uL)
Cacatua galerita	6.6
Burhinus grallarius	44.3
Canis lupis	29.4
Lagorchestes	22.1
Petrogale brachyotis	23.7
Planigale maculata	24.0
Pseudantechinus bilarni	31.1
Pseudantechinus	18.7
Rattus tunneyi	26.9
Tachyglossus aculeatus	160.9
Trichosurus vulpecula	53.4
Zyzomys argurus	23.6
Zyzomys palatalis	33.8
Pseudomys johnsoni	27.3
Wyulda squamicaudata	46.7

3.4.2 Bioinformatics pipeline to read samples

An eDNA analysis pipeline was developed by Omics2View Consulting (Germany) to process and assign taxonomic identities to the raw eDNA sequence data amplified by the pooled Aves02, Mamm02 and Euk, PCR reactions (Appendix 2). Based on the *DADA2* algorithm utilised for bacterial DNA analysis, the pipeline processes and generates Amplicon Sequence Variants (ASVs) from the multiplex-sequence data. Using the IDTAXA Classifier of the DECIPHER package, the pipeline can assign taxonomic identities to sequence data utilising either the publicly available NCBI nucleotide database or the custom-built reference DNA database. Briefly, in 'Analysis mode', the pipeline sorts and cleans the raw sequence read data using BBMap (Bushnell 2020) to locate and remove the primer and adapter residues along with any sequence pairs that did not contain the expected residues. The *DADA2* algorithm then filters and trims sequences successively to remove sequences with fewer than 70 bp. The reads were then filtered and the forward and reverse reads denoised and assembled to construct contigs called ASVs. Assembled ASVs were then compared to a constructed ASV count table and removed if they were of atypical length. Chimeras were removed then taxonomy assigned to the surviving sequences using the IDTAXA Classifier of the DECIPHER package.

The pipeline was run using both NCBI and our internal reference sequences to identify the taxa associated with the ASVs. The analysis was only run at the taxonomic level of genera or above. Counts of the number of detections for each taxon identifier were collated for each sample and associated with location site and/or as a mock community.

The packages (DECIPHER/IDTAXA) used to identify the taxonomy of the sequences in our bioinformatic pipeline are quite conservative, and broadly don't attempt to identify sequences to species level. This is due to a number of studies showing that if researchers use classifiers with fuzzy-matching/machine learning/bootstrapping methods on widely used gene fragments (e.g. 16S), and try to classify these fragments to species-level, there tends to be a high rate of false positives. Mostly these studies have been done on bacteria, so it remains to be seen whether such a result is also true for vertebrate datasets.

Simultaneously, the classifiers used by our pipeline are much more intelligent than the basic NCBI BLAST function. So, while BLAST may give us more species-level identifications, it is also likely to substantially increase our false-positive rate

There was good species recovery using the mammal primers (Table 3.5). Most species were identified in the pure mock-community samples including eDNA environmental water samples spiked with the mock community (42-M-S, 56-M-S, 69-M-S). The mammal primers appear to preferentially bind *P. bilarni* over *P. mimulus* (0 counts) and *Z. argurus* over *Z. palatalis* (low counts). There was also good recovery using the bird primers (Aves02) (Table 3.6).

Table 3.5. Mock community results using theMamm02 primers.

eDNA ID 42-M-S	eDNA ID 56-M-S	eDNA ID 69-M-S	eDNA ID M1	eDNA ID M2	Total # reads	Taxonomy ID	Conf%	Sequence	Tissue library match
18	49	15	49	58	189	Tachyglossus	100	AAACTAATTGAGTAAAAATTTTAGTTATTATACCCTTAAGT TGAATGTTTGAAGAAGTTACTCTTTAATTAA	TDNA 92 (Tachyglossus aculeatus)
23	27	11	37	33	135	Canis	100	AAATTGCTAACTCATAATAATGGTGTTATGTTATGCCTTGT AGGTATCTAGTATCCATAAGTTTGGGTTAGTTAATTAA	TDNA 86 (Canis dingo)
9	25	5	27	23	89	Petrogale	100	TAAGATTCATAGTCTAATTCGAAATATTTACACCCCTAAAT ACAGGAACAAAATCAGACCTACTAGACTATAACCTT	TDNA 88 (Petrogale brachyotis)
38638	57185	45439	71453	69343	283423	Tachyglossus	100	TTAATTAAAGAGTAACTTCTTCAAACATTCAACTTAAGGGT ATAATAACTAAAATTTTTACTCAATTAGTTT	TDNA 92 (Tachyglossus aculeatus)
24956	34424	29458	42245	42347	175036	Canis	100	TTAATTAACTAACCCAAACTTATGGATACTAGATACCTAC AAGGCATAACATAACACCATTATTATGAGTTAGCAATTT	TDNA 86 (Canis dingo)
761	1261	855	1137	1226	5257	Lagorchestes	98	TAAGATTCATAGTCTAATTCAAACAACATTACCCTACTCA CAGGAACAAATCCAGACCTACTAGACTATAACCTT	TDNA 87 (Lagorchestes conspicillatus)
	4				4	Zyzomys	96	AAATTTTGAGCTTAATATTTATTGTGTTATGCCATTAGGTT ATTTAAATATATTAATTAAGCTTTTAATTTA	TDNA 96 (Zyzomys palatalis)
1030	1639	1369	1686	1772	7732	Zyzomys	95	TAAATTAAAAGCTTAATTAATATATTTAAATAACCTAATGG CATAACACAATAAATATTAAGCTCAAAATTT	TDNA 95 (Zyzomys argurus)
41	36	33	61	55	226	Wyulda	94	AAGGTTATAGTCTAGTTGGTTATAGTGTTGTTCCATGTAG GGTTAAATTTGGTTAGATTAGACTATGAATCTTA	TDNA 8 (Wyulda squamicaudata)
2	1	2	6	2	13	Pseudantechinus	88	AAGACTATAACTCAGGTGTTTATACATATTGTTCCTGTAG GTTGAATGTTCGTATGTGTTGAGTTATGTGACTTA	TDNA 94 (Pseudantechinus bilarni)
38910	55299	43160	72706	71135	282392	Wyulda	88	TAAGATTCATAGTCTAATCTAACCAAATTTAACCCTACATG GAACAACACTATAACCAACTAGACTATAACCTT	TDNA 8 (Wyulda squamicaudata)
2023	2646	2218	3745	3625	14295	Pseudantechinus	86	TAAGTCACATAACTCAACACATACGAACATTCAACCTACA GGAACAATATGTATAAACACCTGAGTTATAGTCTT	TDNA 94 (Pseudantechinus bilarni)

5	7	2	8	6	28	Pseudomys	83	AAATTATAAGTTTAGTATTTTTGTGTTGAGCCAATTAGGTTTTGTAGATGCGAAAATTAAACTTTTAATTTA	TDNA 5 (Pseudomys johnsoni)
6220	9815	8529	10670	11432	46851	Pseudomys	83	TAAATTAAAAGTTTAATTTTCGCATCTACAAAACCTAATTGGCTCAACACAAAAATACTAAACTTATAATTT	TDNA 5 (Pseudomys johnsoni)
326	450	312	556	525	2176	Rattus	83	TTAATTTACTAGTTTAACTTATGCATAATAACCTAATGGACCAAAACAACATAATCATAAACTAAAAATTT	TDNA 91 (Rattus tunneyi)
2					2	Planigale	79	AAGACTATAGCTCAGGACTTTATACATTTTTGTCCCGGTAGGTTTAATTAGGTATGTGTTGAGCTATGTGTCTTA	TDNA 89 (Planigale maculata)
366	582	384	722	645	2705	Planigale	77	TAAGACACATAGCTCAACACATACCTAATTAAACCTACCGGGACAAAAATGTATAAAGTCCTGAGCTATAGTCTT	TDNA 89 (Planigale maculata)
8	52	5	46	35	146	Canis	74	TTAATTAACTAACCCAAACTTATGGATACTAAATACCTATAAGGCATAACATAACACCATTATTATGGGTTAGCAATTT	TDNA 86 (Canis dingo)
3	1	4	8	10	26	Trichosurus	71	AAGGTTATAGTCTAGTTAGTCATTGTATTGTTCCATGTAGGGTTTTGTTGAGTTAGGTTAGACTATGAATCTTA	TDNA 93 (Trichosurus vulpecula)
			2		2	Zyzomys	68	AAATTTTGAGTTTAATATTTATTGTGTTATTGCCATTAGGTTATTTAAATATATTAATTAAACTTTTAATTTA	TDNA 96 (Zyzomys palatalis)
44	113	46	109	116	430	Muridae	66	TAAATTAAAAGTTTAATTAATATATTCAAATAGCCTAATGGCATAACACAATAAATATTAAACTCAAAATTT	TDNA 95 (Zyzomys argurus)
	72			52	124	Trichosurus	65	TAAGATTCATAGTCTAACCTAACTAAACAAAACCCTACATGGAACAATACAATGACTAACTAGACTATAACCTT	TDNA 93 (Trichosurus vulpecula)
			76	61	137	Phalangeridae	64	TAAGATTCATAGTCTAACCTAACTCAACAAAACCATACATGGAACAATACAATGACTAACTAGACTATAACCTT	TDNA 93 (Trichosurus vulpecula)
3995	5906	4774	7062	7010	28845	Trichosurus	63	TAAGATTCATAGTCTAACCTAACTCAACAAAACCCTACATGGAACAATACAATGACTAACTAGACTATAACCTT	TDNA 93 (Trichosurus vulpecula)
2445	4224	3081	4152	4415	18394	Zyzomys	62	TAAATTAAAAGTTTAATTAATATATTTAAATAACCTAATGGCAATAACACAATAAATATTAAACTCAAAATTT	TDNA 95 (Zyzomys argurus)
32	27		43	42	144	Mammalia	62	TAAGATTCATAGTCTAACCTAACCCAACAAAACCCTACATGGAACAATATAATGACTAACTAGACTATAACCTT	TDNA 93 (Trichosurus vulpecula)
		22			22	Trichosurus	61	TAATATTCATAGTCTAACCTAACTCAACAAAACCCTACATTGAACAATACAATGACTAACTAGACTATAACCTT	TDNA 93 (Trichosurus vulpecula)
14	13	7	36	33	103	Pseudantechinus	60	TAAATCGCATAACTCAACACATACGAACATTCAACCTACAGGAACAATACGTATAAACACCTGAGTTATAGTCTT	TDNA 94 (Pseudantechinus bilarni)
62	121	28	101	87	401	Zyzomys	60	TAAATTAAAAGTTTAATTAATATATTTAAACAGCCTAATGGCATAACACAATAAATATTAAACTTAAAATTT	TDNA 95 (Zyzomys argurus)

	36				36	Mammalia	60	TAAGACACATAGCTCAACACATACTTAATTAAACCTACCG GGACAAAAATGTATAAAGTTCTGAGCTATAGTCTT	TDNA 89 (Planigale maculata)
17	39	2	33	27	118	Dasyuridae	60	TAAGTCGCATAACTCAACACATACGAACATTCAACCTACA GGAACAATACGTATAAACACCTGAGTTATAGTCTT	TDNA 94 (Pseudantechinus bilarni)

Table 3.6. Mock community results using the Aves02 primers.

eDNA ID	eDNA ID	eDNA ID	eDNA ID	eDNA ID	Total # reads	Taxonomy ID	Conf%	Sequence	Tissue library match
42-M-S	**56-M-S**	**69-M-S**	**M1**	**M2**					
170	122	74	245	244	855	Burhinus	100	CTCACCTCACTTGAGAGCACATCAGTGAGCACAATAGCCCAACCCGCTAGCAAGACAGGTCAAGGTATAGCCAATGGGAGC	TDNA 85 (Burhinus grallarius)
11967	12261	8386	39849	43329	116560	Burhinus	100	GCTCCCATTGGCTATACCTTGACCTGTCTTGCTAGCGGGTTGGGCTATTGTGCTCACTGATGTGCTCTCAAGTGAGGTGAG	TDNA 85 (Burhinus grallarius)
1279	1309	1091	4860	4850	13453	Cacatua	75	ACTCCATAGGCTATACCTTGACCTGTCTTGTTAGTGGTTGTGGACTATTGGGCTCACTGTTGTGCTTTCATAAAGGTGAG	TDNA 84 (Cacatua galerita)
24	10	10	27	33	104	Cacatua	75	CTCACCTTTATGAAAGCACAACAGTGAGCCCAATAGTCCACAACCACTAACAAGACAGGTCAAGGTATAGCCTATGGAGT	TDNA 84 (Cacatua galerita)

3.5 Validating the approach using waterhole samples

Water samples were collected from 13 sites on Wollogorang Station in September 2019. In the MacDonnell Ranges, 22 waterholes were sampled in the East MacDonnell Ranges, Ormiston Gorge and Simpsons Gap in November 2020. Sites were selected that were known to have had records of Ccntral rock-rat, brush-tailed possums and black-footed rock-wallabies. Due to the threatened status and decline of the target mammal species in central Australia, the sampling strategy was to sample broadly across in the ranges in the hope of detecting these significant species.

Optimal timing for field sampling was uncertain. The two best options were after first-flush rains, potentially having a higher DNA load washed in, or later in a dry period when the concentration of any DNA in the residual water is likely to be more concentrated. The dry period was chosen due to logistical constraints in accessing Top End sites in the wet season, the potential for broader distribution of DNA with larger volumes of water washing through gorges and the unpredictability of the timing of rain in central Australia.

Each waterhole was photographed, and conditions were described, including approximate measurements of the waterhole. One negative control sample was collected at each site. A 250 mL Nalgene bottle filled with approx. 200 mL of HP water had the lid removed and was exposed to the air for 2 minutes, sealed, and stored at 4^{o}C. Water samples were collected by submerging a 250 mL Nalgene bottle and collecting 200 mL of water. Sampling location within the waterhole was chosen where water was still and to reduce any collection of algae. An additional 400 mL of water was collected *in situ* for the collection of physiochemical measurements within 24 hours of collection. Samples were stored within cool bags with ice-bricks and transferred to a refrigerator to be stored at 4^{o}C as soon as possible. Samples were transported to the lab for filtering. Samples were filtered within 12 hours of collection.

Samples were processed as described for the mock community (Appendix 3). Sequence reads were rarely above 50 and were not different from the negative controls (examples shown in green). In contrast, the mock library samples analysed alongside these water samples had high sequenced reads (shown in orange). As a consequence, these data were not analysed further and reasons for this are raised in the discussion.

4. Discussion

In this proof-of-concept project, we aimed to develop a multi-species approach to detecting terrestrial animals for northern Australia. A tissue library initiated in this project is being maintained and an approach outlined in Figure 4.1 below summarises the approach taken. The primers Mamm02 and Aves 02 were successfully used to recover a mock community but this approach has yet to be successfully operationalised. Although an attempt was made to validate the approach using environmental water samples, it is likely that the small volume (250 mL) of water was insufficient to collect sufficient DNA to detect the species present. It is likely that 2–10 L may be required for reliable detection. Given this outcome and the reality of transporting large volumes of water, it may be better to focus detection on a small number of threatened species and take a single-species qPCR approach. A possible approach is to use eDNA metabarcoding as a first pass, then to develop single-species tests for organisms of interest, particularly if invasive species detection is the goal.

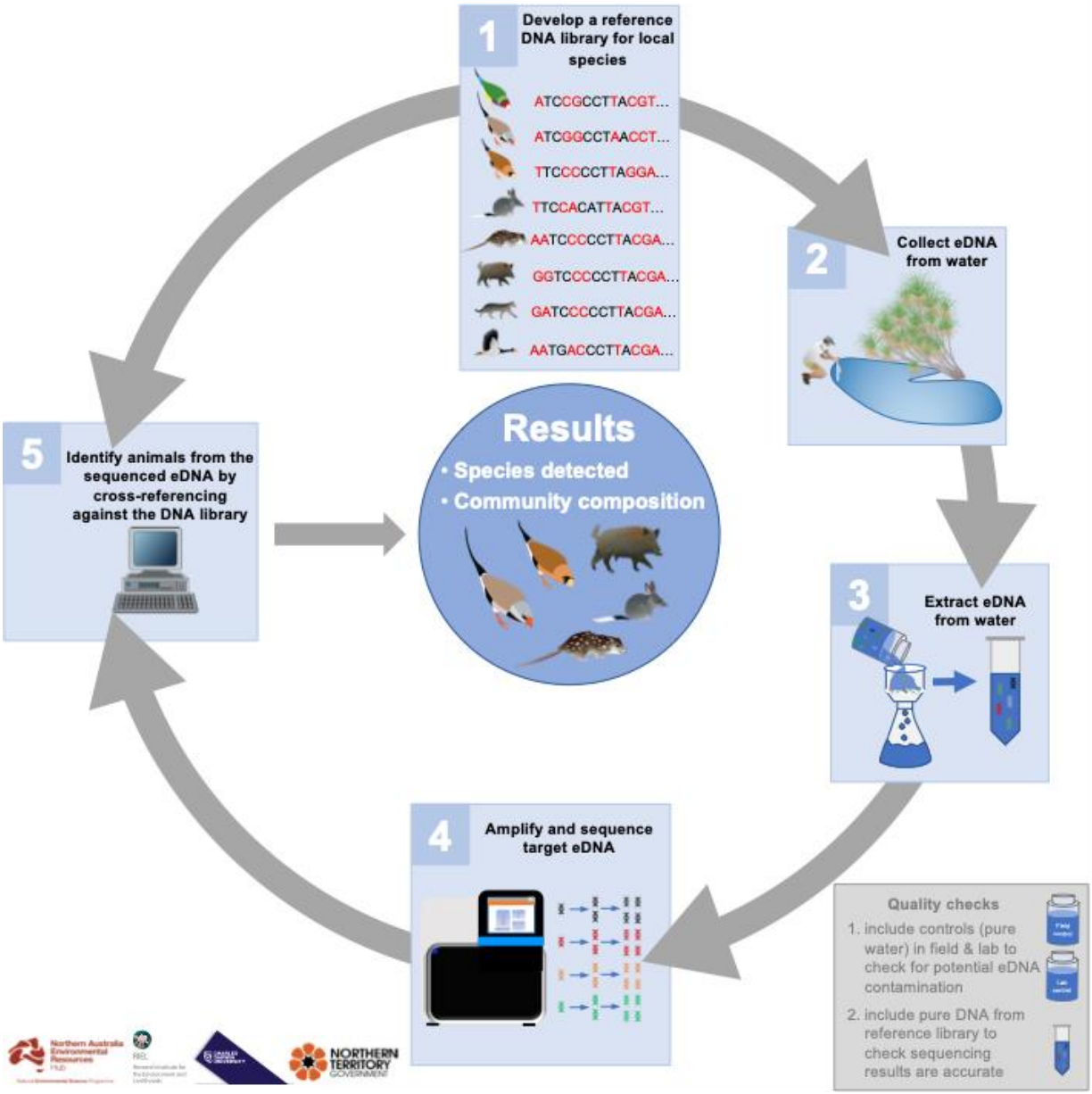

Figure 4.1. eDNA process overview. Symbols courtesy of the NESP Northern Australia Hub, nespnorthern.edu.au and Integration and Application Network (ian.umces.edu/media-library).

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Appendix 1

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ENDANGERED SPECIES RESEARCH
Endang Species Res

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Development and validation of an environmental DNA test for the endangered Gouldian finch

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ABSTRACT: Detecting animals by identifying their DNA in water is a valuable tool for locating and monitoring species that are difficult to detect through other survey techniques. We developed a test for detecting the endangered Gouldian finch *Erythrura gouldiae*, a small bird endemic to northern Australia. Only 1 previous study has reported an environmental DNA (eDNA) test that unequivocally identifies a bird species using the water bodies from which they drink. In controlled aviary trials with a pair of Gouldian finches, first detection in 200 ml of water occurred after as little as 6 h, but the detection rate was higher at 30 h. DNA persisted in water exposed to the sun for <12 h and in the shade for 12 h. In trials with 55 finches, persistence was up to 144 h. The eDNA test for finches and the Gouldian finch-specific test were positive for waterholes where Gouldian and other finch species were observed each morning over 3 d. Importantly, where no Gouldian finches were observed for up to 72 h prior to water sampling, the Gouldian test was negative. Where other species of finch but no Gouldian finch were observed and counted, the finch test was positive, but the Gouldian finch test was negative. This approach could be developed for broad-scale monitoring of this endangered species, and potentially applied to a much broader range of terrestrial species that shed DNA into water bodies.

KEY WORDS: Birds · Distribution · eDNA · Filter · qPCR · Water · Gouldian finch · *Erythrura gouldiae*

1. INTRODUCTION

Bodies of fresh water are a useful resource for studies of vertebrate presence, absence and overall distribution inferred by detection of their DNA (Deiner et al. 2015, Fukumoto et al. 2015). The use of environmental DNA (eDNA) for detection of individual species has been applied to many aquatic animals such as amphibians (Fukumoto et al. 2015), reptiles (Piaggio et al. 2014), fish (Thomas et al. 2016) and insects (Furlan & Gleeson 2017). The detection of non-aquatic vertebrates requires a pathway for DNA to be shed into the water. Cells or extracellular DNA deposited when drinking or defecating can in theory be used to identify animal presence at or near water bodies. There is some evidence that the presence of terrestrial mammals can be determined by sampling the water bodies from which they drink (Ushio et al. 2017). Only 1 study on eDNA detection of live, non-aquatic birds from water has been published to date (Ushio et al. 2018).

The Gouldian finch *Erythrura gouldiae* is a brightly coloured passerine bird in the family Estrildidae (Tidemann 1996, Bolton et al. 2016). The species has a broad and discontinuous distribution across the savannah woodland of northern Australia (Fig. 1) from Cape York to the western Kimberley region (O'Malley 2006). The Gouldian finch is currently listed as 'endangered' in Australia (Environment Protection and Biodiversity Conservation Act of 1999)

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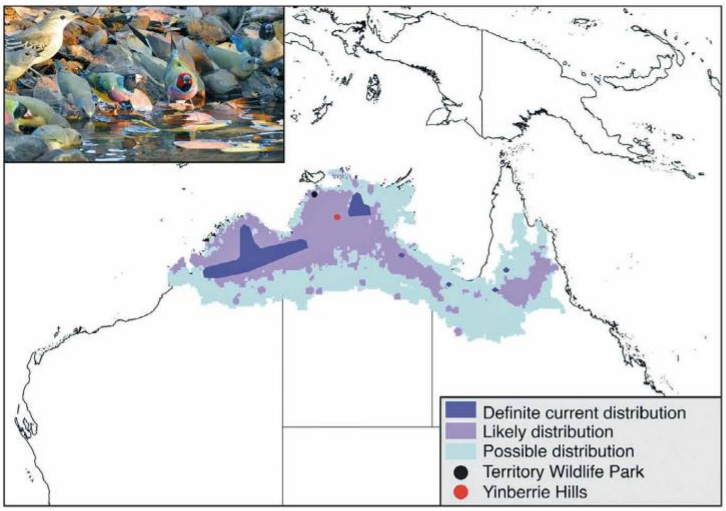

Fig. 1. Distribution of *Erythrura gouldiae* in northern Australia (Atlas of Living Australia, download at www.ala.org.au) with an inset picture of birds drinking from surface water. Photo: Alexander and Eckhard Garve, Germany

due to habitat loss and degradation across its range (Dostine et al. 2001, O'Malley 2006). The International Union for the Conservation of Nature lists *E. gouldiae* as Near Threatened (Rodrigues et al. 2006). Current methods for detection and monitoring the Gouldian finch are fairly resource intensive and impractical to apply across the entire population.

The Gouldian finch is an ideal candidate for testing eDNA detection of a terrestrial species in water. Their primarily granivorous diet requires finches to drink water on a daily basis, providing a pathway for DNA to shed into the environment. During the late dry season in the monsoonal tropics, the release of Gouldian finch DNA into drinking water becomes more concentrated as waterholes suitable for drinking become rare (Evans et al. 1985). Mixed bird flocks drinking at such waterholes often also include long-tailed finch *Poephila acuticauda* and masked finch *P. personata*, as well as other members of the Estrildidae. Therefore, the eDNA test for Gouldian finches needs to be specific enough to differentiate between these closely related species.

Quantitative PCR (qPCR) assays have been demonstrated as a sensitive approach for detection of animals from eDNA (Thomsen & Willerslev 2015). The qPCR assay system we designed uses a group-specific PCR primer set to amplify a fragment of mitochondrial DNA from birds in the family Estrildidae and other close relatives. The sequence of this amplicon is variable among species, but contains a short region that is specific to the Gouldian finch. We designed a hydrolysis probe for this region that allows detection of Gouldian finch DNA, but not DNA from close relatives that are sympatric with the Gouldian finch. This hybrid group-specific PCR detection system also allows for the use of spike-in positive control DNA (Furlan & Gleeson 2017). This system for detecting Gouldian finch DNA is very effective for this species and provides an example of a robust PCR-based assay for eDNA with effective internal positive controls and a carefully established detection sensitivity.

We describe the development of this detection system in detail. We used controlled conditions in a captive environment (aviary) to trial and refine protocols for water sampling in the field. We report the effectiveness of the approach to detect Gouldian finch eDNA in a wild/natural environment.

2. MATERIALS AND METHODS

2.1. Library construction, sequencing and isolation of sequences of interest

Total DNA was purified from the blood of 3 finch species (Gouldian, long-tailed and masked) with a DNeasy Blood and Tissue DNA extraction kit (Qiagen). The DNA was used for Illumina library construction and sequenced in 150 bp uni-directional reads on a NovaSeq platform (NovaSeq6000, 2 × 150 bp flowcell, SP chemistry; Ramaciotti Centre, Sydney, Australia). Three individuals from each species were analysed to make a total of 9 libraries. Two regions of interest were extracted from the pools of shotgun-sequenced finch DNA: the nuclear ribosomal DNA

gene regions and the mitochondrial DNA control region. These regions were identified in the libraries by choosing a reference sequence from a near relative and aligning reads on it with Bowtie2 (Langmead & Salzberg 2012). The reference sequences for the nuclear ribosomal DNA region were from *Goura cristata* (GenBank MG590307.1) and for the control region from the zebra finch *Taeniopygia guttata* (GenBank DQ43515.1). Contigs were generated from the sequences that aligned with the reference sequences using Spades (Bankevich et al. 2012). Homologous contigs were then aligned with ClustalX for visualisation and primer design (Thompson et al. 2002)

2.2. Amplification PCR primer design

Primer-binding sites were manually selected from the aligned sequences for each region. Candidate pairs primer of binding regions were tested in Oligo-Calc (http://biotools.nubic.northwestern.edu/OligoCalc.html) for primers with compatible annealing temperatures and lack of self-complementarity (Kibbe 2007). Each primer-binding site was tested by BLASTn (Altschul et al. 1990) against the current non-redundant database from GenBank to identify any potential sequence variants that would affect primer binding. Amplification primer-binding sites were chosen that were specific to Estrildidae finches, including *Erythrura* and *Poephila*, and in a group-specific approach would produce a small enough PCR fragment for degraded DNA templates (Deagle et al. 2006) that had species-specific sequences within it (Jarman et al. 2004). This approach reduces the chance of false negative amplifications if Gouldian finch individuals had mutations in the primer-binding sites. Amplification primers (Table 1) and the Gouldian finch probe were synthesised by IDT Technologies.

Table 1. DNA sequences for the Gouldian finch *Erythrura gouldiae* eDNA detection assay. HEX and TET fluorescent 5′ modifications are quenched by the Iowa Dark quencher (IDQ) when the probe is not digested by *Taq* polymerase. In contrast, the probe is digested and fluorescence occurs when it binds to target *E. gouldiae* sequence present in a positive test. All sequences are presented in the 5′-3′ orientation

DNA region	Sequence
E. gouldiae control region fragment. Identical to GenBank KX858952.1, KX858951.1, EF094912.1	TCA GGT ACC ATA CAG CCC AAG TGA TCC TAC CTC CGG CCA GAG CCG CAA GCG TCA CCC GTA ATG CTA GGG ACT TAT CTA TTG TAC TTA CTC CTT AAC CTG GGA AAC GAC TAA TGT CAC AGT ACT CCT TTG CAT TCC TAA GGT CTA TGG AAT TCG CCC ACC TCC TAG G
Poephila acuticauda control region fragment. Identical to GenBank JQ255410.1, JQ255410.1	TCA GGT ACC ATA TAG CCC AAG TGA TCC TAC CTA CAG GCC GAG CCG CAA GCG TCA CCC ACA CAC ATA GGA ACT TTC CAT TGT ACT AAA CCC CCA ACC CAG GAA ACG AGG AGT GTT ACT GTA CAC CTT TGC ATT CTC AAA GTC TAC TGA ATT CGC CCA CCT CCT AGG
Poephila personata control region fragment	TCA GGT ACC ATA TAG CCC AAG TGA TCC TAC CTA CAG GTC GAG CCG CAA GCG TCA CCC ACA CAC ATA GGA ACT TTC CAT TAT ACT AAA CTC TCA ACC CAG GAA ACG AGG AGT GTT ACT GTA CAC CTT TGT ATT CTC AAA GTC TAC TGA ATT CGC CCA CCT CCT AGG
Synthetic positive assay control	CAG ACA ACA TTC AGG TAC CAT ACA GCC CAA AAG TCT TTC CCT CTT TCG CGT CAT GCC GCT GGG GAA TCC CAA ATA TCC GCA AAT ATG GAG GCG ACG GTC TAA TCC TCC TTT CCG AAC CCC CCG CAT TCT CCT TCA GAT TTT CAT GGA ATT CGC CCA CCT CCT AGG CTC GCT AGA C
FinchCR_F1 Finch control region forward primer	TCA GGT ACC ATA YAG CCC AA
FinchCR_R1 Finch control region reverse primer	CCT AGG AGG TGG GCG AAT TC
Gouldian finch probe *E. gouldiae* internal hydrolysis probe	HEX-CGT CAC CCG TAA TGC TAG GGA-IDQ
DNA positive probe Synthetic positive control internal hydrolysis probe	TET-GTC TAA TCC TCC TTT CCG AAC C-IDQ

2.3. Design of a species-specific probe

A hydrolysis probe was designed to identify amplicons that came only from the Gouldian finch. The sequence for the probe was identified from an alignment of the nuclear control region of sequence data from the 3 species of finch. We also searched GenBank for sequences from related finches. This search produced 38 hits for the Gouldian finch which were produced in a previous population genetics study (Bolton et al. 2016). The hydrolysis probe was labelled with the fluorophore HEX at the 5′ end and the Iowa Dark quencher at the 3′ end (Table 1). Amplification primers and probes were tested for specificity to group or species by BLASTn searches of GenBank (Altschul et al. 1990).

2.4. Design of an internal positive control synthetic DNA fragment

A fragment of synthetic DNA was synthesised (IDT Technology) for use as an internal positive control for the PCR amplification of environmental DNA (Furlan & Gleeson 2017). The GC% of the Gouldian finch DNA was determined, and randomly generated fragments of DNA with similar GC% and length were assessed by BLASTn (Altschul et al. 1990) searches of GenBank to ensure that they had no unintended homology to any known organisms that might also be present in eDNA.

2.5. Water sampling and eDNA purification

Water samples (200 ml) were collected from water bodies with sterile Nalgene bottles. The water was filtered through sterile, 0.45 µM, mixed cellulose ester filters (Merck Millipore). DNA was purified from the filters using a DNeasy Blood and Tissue DNA extraction kit (Qiagen). Samples were extracted according to the manufacturer's guidelines for tissue (step 1a) with some modifications. Before extracting, filters were placed into sterile, 2 ml freestanding screw tubes containing 4 sterile, 2 mm glass beads. Filters were pulverised into 2–3 mm fragments by immersing the tubes in liquid nitrogen and disrupting with a Mo Bio PowerLyser™ 24 at 800 × *g* for 30 s. For each sample, the volumes of lysis and buffer solutions (Quiagen) were 60 µl Proteinase K, 540 µl buffer ATL, 600 µl buffer AL and 600 µl 100% ethanol. During lysis, filters were incubated overnight at 56°C in a BIOLINE Shaker. Purified DNA was eluted in 200 µl of H_2O. The yield and quality of extracted DNA was checked with a 1 µl subsample using a NanoDrop 2000 (Thermo Scientific).

2.6. Molecular sensitivity testing for the Gouldian finch DNA detection assay

The number of Gouldian finch mitochondrial molecules that the assay could detect was quantified by diluting extracted Gouldian finch DNA over 8 orders of magnitude (from 100 picomoles to 10 attamoles) with 10 mM Tris-HCl pH 7.5. Purified Gouldian finch DNA was extracted from 10 µl of nucleated blood using the DNeasy Blood and Tissue Kit. Each dilution was mixed with Quantitect SYBR Green (Qiagen) and 0.1× BSA, and then run in triplicate on a Rotor-Gene® Q (Qiagen).

2.7. Test validation using captive birds

Aviary trials were conducted at the Territory Wildlife Park (TWP), 45 km south of Darwin, Northern Territory. Trials were run to determine optimal sample volume, time until first detection, eDNA persistence, turbidity effects and species specificity. For each trial, 18 l trays of tap water were exposed to either a single pair (2 individuals; aviary dimension 3 × 1.2 × 2 m^3) or a large group (55 individuals; aviary dimension 8 × 5 × 3 m^3) of Gouldian finches over 3 consecutive days. Each aviary was fitted with a surveillance camera to monitor interactions with the water tray. Each trial was repeated 3 times, and negative controls were collected at the start of each trial, with the exception of the specificity trial. Water trays were placed away from roosts to avoid droppings. Water samples were collected in triplicate using sterile gloves, syringes and bottles.

To determine the optimal sample volume, 20, 50, 100 and 200 ml samples were collected after 3 d of exposure to a single pair of Gouldian finches. Cameras showed that the birds became active after 07:00 h, so trials started at 07:00 h. To measure first detection of eDNA from a single pair of Gouldian finches, 200 ml samples were collected at 12:30 h on the day of fresh water exposure, to represent half daily activity. A second sample was collected the following morning at 06:45 h, before the birds became active, to capture the whole activity period of the previous day. Aviary waters were sampled at 12:30 and 06:45 h over 3 consecutive days of exposure to Gouldian finches. To measure persistence of eDNA, aviary water was transported from TWP to Charles Dar-

win University (CDU) after 3 d of exposure to the large group of Gouldian finches. This water was placed in direct sunlight, and 200 ml were collected in triplicate at 0, 12 and 24 h (since commencement of trial) of the first day and every 24 h thereafter, until there was no more water. The trial was repeated for a single pair of Gouldian finches, with the addition of a shaded treatment (double layer of 80% shade cloth).

To test the effect of turbidity, sterilised sediment (<63 µm) was used to simulate turbid conditions, and turbidity was measured with a Lovibond turbidimeter, in nephelometric turbidity units (NTU). Treatments were water (10 l tap water and 5 l homogenised aviary water) and turbid (as for water treatment but with sediment added to 100 NTU). Treatments were left in direct sunlight, and 200 ml were collected in triplicate at 0, 12 and 24 h (since commencement of trial) of the first day and every 24 h thereafter, until there was no more water. To test for inhibition from the turbid DNA samples, 2 positive samples comprising 1 µl of Gouldian finch DNA from blood samples were spiked with 1 µl of DNA extracted from the 'turbid' samples. To account for dilution, 1 µl of high pure water was added to 2 other positive control blood samples.

Specificity was tested using water from 3 aviaries at the TWP that contained a range of bird species, including closely related sympatric species but no Gouldian finches. The aviaries were (1) Pandanus Creek, containing crimson finches *Neochmia phaeton*, (2) Paperbark Swamp, containing long-tailed finches and chestnut-breasted mannikins *Lonchura castaneothorax* and (3) Walk Through Aviary, containing a mix of other bird species but no finches of any species. Water samples were collected from the artificial pools in each aviary.

2.8. Statistical tests

To determine which factors significantly influenced eDNA detection, we applied permutational testing using Primer7 software (Anderson et al. 2008). To quantify similarities between samples, we used a Bray-Curtis similarity matrix (detection positive = 1 and negative = 0) and zero-adjusted (Clarke et al. 2006). Raw data were not transformed because the dataset consisted of 1 and 0 values only. Data were analysed using a PERMANOVA with 999 permutations. Results were considered significant at $p < 0.05$. Each experiment was analysed using 2 or 3 factors (see Table A1 in the Appendix for factor allocations). Briefly, experimental factors included aviary, volume, visits, sampling round, temperature and treatment. Turbidity and specificity experiments did not require statistical analyses because they were all negative and had small sample numbers, respectively.

2.9. Test validation at waterholes

Water was sampled as described above from small natural waterholes at the Yinberrie Hills (45 km north of Katherine, Northern Territory), which supports the largest extant breeding population of Gouldian finches in the Northern Territory (Dostine et al. 2001). Waterholes were selected following a site visit in early September 2018 to assess access, water levels and use by birds. The aim was to sample 2 waterholes in each of 3 categories: high, moderate and no bird usage. Six waterholes were selected (see Table 3 for their locations and sampling dates). Birds were counted at each waterhole to provide an estimate of recent use by all finch species and to test the robustness of both the general finch and Gouldian-specific eDNA tests. The number of Gouldian, long-tailed and masked finches were counted at each waterhole between 07:00 and 10:00 h for 3 consecutive mornings and prior to sampling.

To determine whether local environmental conditions affected the probability of eDNA detection, water samples were collected from 3 locations within each waterhole. Three 200 ml water samples were taken 'at' (0 m), 'near' (~0.5 m) and 'far' (~2 m) from where birds were observed drinking for each waterhole. Where no birds were observed drinking, sampling locations were taken from the edge most representative of a typical drinking point. Sampling included 1 negative control where a 250 ml Nalgene bottle filled with high pure water was opened for 2 min, closed and stored at 4°C. At each site, we also collected 6 water samples (each 200 ml) for a 2 wk storage trial at 4°C (3 samples site^{-1}) and at room temperature (3 samples site^{-1}). This was done to assess robustness of the test when filtering equipment was not available in the field and samples had to be stored for an extended period before processing. Waterhole volume was estimated by measuring length, width and depth at multiple points using a tape measure.

3. RESULTS

3.1. Shotgun DNA sequencing

Between 1.2 and 1.45 million sequence reads were generated for each genome skimming library (Dods-

worth 2015). Contigs were assembled for both the control region and internal transcribed spacer 2 (ITS2) regions for 3 individuals of each species of Gouldian, long-tailed and masked finches. Sequence variation in the control region contigs among all 3 species was identified and incorporated into primer designs by either avoiding the variable sites, or by using degenerate base sites to accommodate the identified variants.

3.2. Gouldian finch eDNA qPCR detection assay validation

The PCR-based detection system that we designed and tested for Gouldian finch environmental DNA is shown in Fig. 2. Amplification specificity was tested empirically at a range of different annealing temperatures from 45 to 60°C. Amplification products were separated by electrophoresis at 5 V cm^{-1} on 2% agarose Tris-borate-EDTA gels and visualised by fluorescence at ~520 nm of SYBR Safe (Molecular Probe) under ~460 nm excitation. PCR products of the expected size were observed at all tested annealing temperatures for the control region primer pair. The ITS2 primer pair failed to amplify under these conditions, which is likely because of the very high GC% of the target region, and we subsequently abandoned development of this potential marker.

Thermal cycling conditions for the control region primer pair were optimised at 94°C for 20 min; 45 cycles of 94°C for 10 s, 60°C for 30 s, 72°C for 30 s with optical data acquisition; and 72°C for 2 min. Melt curves were tested for their ability to discriminate among amplicons from Gouldian, long-tailed and masked finches, but the melting profiles were too similar for reliable discrimination. Consequently, a hydrolysis probe was used instead to specifically detect the Gouldian finch. Addition of this probe to the mix produced a species-specific test for the Gouldian finch. The amplification primers and probe region were conserved in the sequences produced by Bolton et al. (2016), so we know that in 41 of 41 examined cases *in silico*, this assay would specifically identify Gouldian finch DNA. Empirical testing of this amplification primer set and probe combination on 3 individuals of each finch species tested only resulted in HEX fluorescence at 556 nm for Gouldian finch DNA. Further testing for amplification from multiple individuals revealed amplification in all 22 cases, suggesting that this test works consistently within the species. No HEX fluorescence was detected for 21 long-tailed or 5 masked finch DNA samples, although SYBR Green fluorescence was, indicating that the

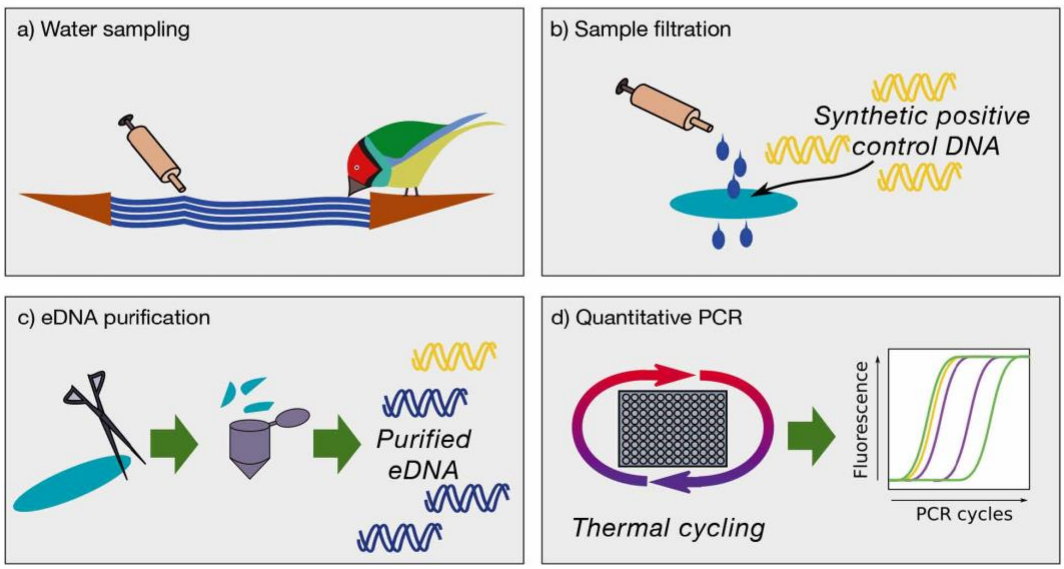

Fig. 2. Gouldian finch eDNA detection assay. Water body where the Gouldian finch might be present is (a) sampled and (b) filtered. Synthetic positive control DNA can be added so that false negative tests can be identified later. (c) eDNA is purified from the filters and (d) analysed by quantitative PCR

group-specific primers amplified from these species. The different types of results that the test can produce are shown in Fig. 3.

The control region fragment of the Gouldian finch that we sequenced and amplified was 166 bp long with a GC content of exactly 50 %. The synthetic positive spike-in control DNA that we synthesised also had a GC content of 50 % and a randomly generated sequence between the primer-binding sites for primers FinchCR_F1 and FinchCR_R1 (Table 1). This was 186 bp long because 10 bp extensions were added to the 5′ and 3′ ends of the primer-binding sites to ensure that exonuclease activity did not affect the amplifiability of the fragment.

3.3. Molecular sensitivity of the Gouldian finch DNA detection assay

Our qPCR assay had a detection limit of 10 attamoles (~300 molecules of 160 bp ds (double-stranded) DNA at 90 kD) of Gouldian finch control region DNA in a qPCR. This level of sensitivity is consistent with that reported for other qPCR assays (Ahrberg & Neužil 2015).

3.4. Gouldian finch detection in aviaries

Gouldian finch eDNA was successfully detected in drinking water from aviaries using our test. The

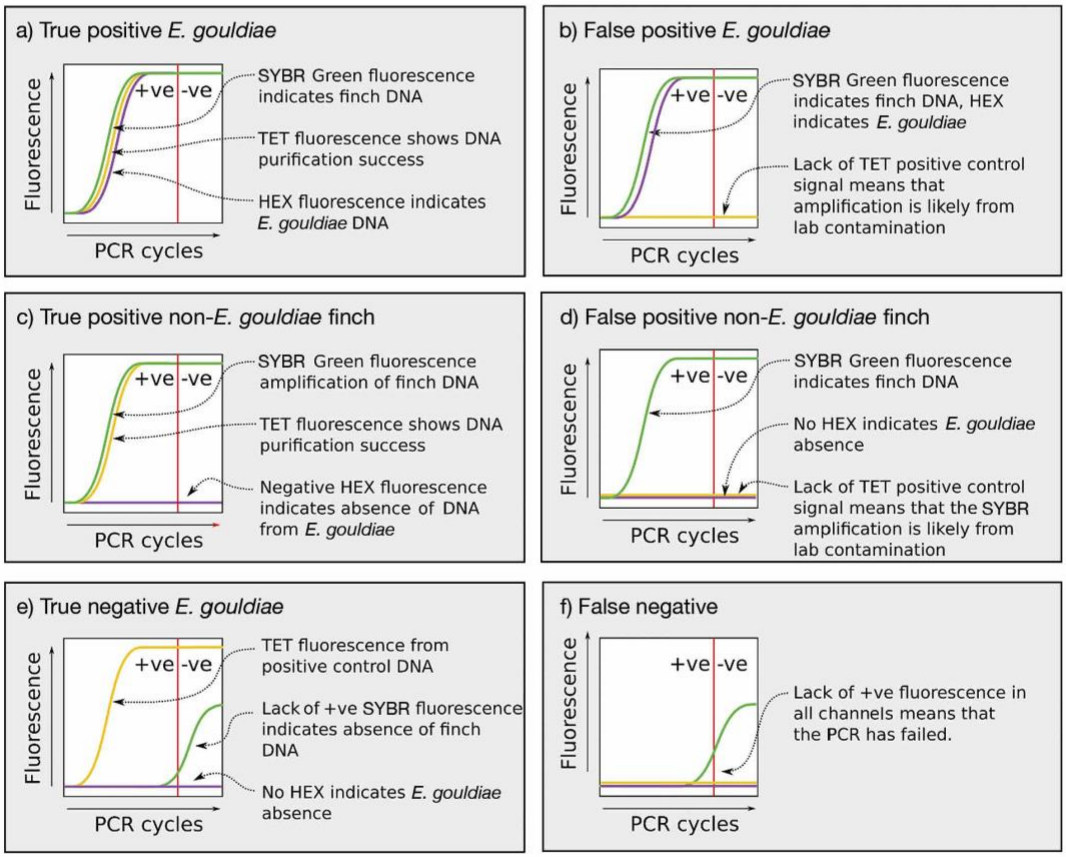

Fig. 3. Interpretation of quantitative PCR results from the Gouldian finch eDNA detection assay. A cycle threshold of 40 cycles (red vertical line) is used to differentiate between positive (+ve) and negative results (-ve). The test includes internal positive control DNA that can be identified through TET fluorescence, which helps to identify false negative and positive results. The Gouldian finch *Erythrura gouldiae* can be identified specifically by HEX, and Estrellidae finches can be identified by SYBR Green fluorescence. If desired, positive tests for these finches could be identified to species level by sequencing the amplicons produced by the qPCR. (a) True positive and (b) false positive *E. gouldiae* test; (c) true positive and (d) false positive non-Gouldian finch test; (e) true negative *E. gouldiae* test; (f) false negative test

volume which resulted in the greatest number of detections was 200 ml ($p = 0.005$), although all sample volumes, including 20 ml, had positive detections (Table 2). First detection was after 6 h of exposure in 1 single-pair aviary. By midday the following day (30 h of exposure), all aviaries had positive detections (Table 2). First detection may therefore occur as soon as 6 h or as late as 30 h following exposure.

Camera surveillance in aviaries showed that the finches drank from the water trays but did not bathe in them. Feathers and a small number of faeces were observed in the water trays, providing DNA from multiple sources. Water trays were visited 11 times d^{-1} on average in the single-pair aviaries and 238 times d^{-1} on average in the large (55 bird) group aviary. Detection was positively associated with 'visits' ($p = 0.001$; Table A1), as expected, since more visits increase the opportunity for DNA to be shed in the water. In the large group aviary, there was an effect of 'aviary' in each repetition of the trial; this is possibly because the birds became familiar with the new watering station and visited more often in the second and third trial run compared to the first ($p = 0.001$; Table A1).

For single pairs of birds, the DNA persisted for 12 h under shaded conditions and <12 h in direct sunlight. However, from the aviary containing 55 Gouldian finches, DNA persisted up to 144 h in direct sunlight. The turbid treatment samples were all negative, and the non-turbid (water) treatment samples were all positive (Table 2, showing time 0 only). The 4 positive control samples, 2 of which were spiked with DNA from the turbid water treatment, were positive, suggesting no inhibition (Chung 2004, Opel et al. 2010).

Water collected from the Pandanus Creek aviary, containing crimson finches, and Paperbark Swamp aviary, containing long-tailed finches and chestnut-breasted mannikins, were positive for the finch test but negative for the Gouldian test (Table 2). Walk Through Aviary, which contained a mix of bird species but no finches, was negative for both tests (Table 2).

Table 2. Aviary trials for detection of finch eDNA. The 9 samples refer to 3 replicates from each of 3 trials per test. For statistical significance values, see Table A1 in the Appendix

Trial	Treatments	Gouldian finch test no. positive / 9	
Minimum sample volume (ml)	200	9	
	100	8	
	20	5	
	50	5	
First detection (h post exposure)	6	1	
	9	3	
	24	2	
	30	7	
Persistence in sun (55 Gouldian finches; h)	0, 12	9	
	24	8	
	48	4	
	72	3	
	96, 120, 144	1	
	168	0	
Persistence in sun (2 Gouldian finches; h)	0	7	
	12	0	
Persistence in shade (2 Gouldian finches; h)	0	8	
	12	1	
	24	0	
Water turbidity	Turbid	0	
	Non turbid	9	
		Finch test	Gouldian test
Specificity	Pandanus Creek	3/3	0/3
	Paperbark Swamp	2/3	0/3
	Walk Through Aviary	0/3	0/3

3.5. Test validation at waterholes

Non-specific finch and Gouldian finch eDNA was detected in natural waterholes at Yinberrie Hills (Table 3). At the 2 waterholes where no finches were observed (JC05 and Deadwomans 4), both the finch and Gouldian finch tests were negative. At 3 waterholes where birds had visited within 24 h of sampling (Poachers, JC10 and Deadwomans 2), the proportion of samples that were positive for the finch test were 100, 89 and 33%, respectively (Table 3), with the 33% result from a large (55 000 l) waterhole. The proportion of samples that were positive for Gouldian finch at those same waterholes were 100, 44 and 0%, respectively. At Deadwomans 1 waterhole where no Gouldian finches had visited within 48 h, but other finch species had (in low numbers), the finch test had 11% positive samples and the Gouldian finch test had none.

Stored water samples from Poachers and JC10 waterholes that were positive when filtered on site were also positive for both the finch and Gouldian finch

Table 3. Bird counts from 3 d of monitoring. GF: Gouldian finch; LT: long-tailed finch; M: masked finch. **Bold** numbers indicate counts made on days when water was sampled. Water samples were collected from 3 locations within each waterhole (at [0 m], near [~0.5 m] and far [2 m] from where birds were observed drinking). Test results are based on a compilation of these drinking site samples

Waterhole name	Latitude (°S)	Longitude (°E)	Estimated volume (l)	Site description at time of collection	Bird counts									Test result	
					Day 1			Day 2			Day 3				
					GF	LT	M	GF	LT	M	GF	LT	M	Finch	GF
Poachers	14° 07′ 24.4″	132° 03′ 40.2″	30 000	Full sun; birds drank from both beaches	157	68	57	**379**	**210**	**52**	227	71	30	9/9	9/9
JC05	14° 07′ 22.8″	132° 03′ 44.2″	380	Full sun; shallow	0	0	0	0	0	0	**0**	**0**	**0**	0/9	0/9
JC10	14° 07′ 24.4″	132° 03′ 33.4″	500	Full shade; film on water surface	472	56	27	59	12	27	**27**	**14**	**8**	8/9	4/9
Deadwomans 1	14° 11′ 09.3″	132° 04′ 57.1″	640	Full sun	60	32	28	0	3	4	**0**	**25**	**0**	1/9	0/9
Deadwomans 2	14° 11′ 06.7″	132° 04′ 56.8″	55 000	Full sun; goshawk present	111	92	72	0	2	0	**61**	**90**	**35**	3/9	0/9
Deadwomans 4	14° 10′ 54.0″	132° 04′ 56.8″	750 500	Dappled sunlight; goshawk present	0	0	0	0	0	0	**0**	**0**	**0**	0/9	0/9

tests after storage for 14 d, regardless of the storage temperature. At Deadwomans 2 waterhole which was 33% positive for the finch test and negative for the Gouldian finch test, all stored samples were negative. At JC05 and Deadwomans 4 waterholes which were negative for both on-site tests, there was a single positive for the finch test (from 3 samples), but the Gouldian finch test remained negative.

4. DISCUSSION

Environmental DNA analysis has become a valuable tool for studying animal distributions (Ushio et al. 2017). It is particularly valuable for detecting animals that are difficult to detect directly by other methods, due to being cryptic, rare, transitory, trapshy or occurring in environments that are difficult or dangerous to sample. The Gouldian finch is a highly mobile species that occurs sparsely over a large area. Obtaining adequate detection probabilities from standard visual census techniques, for either distribution assessment or population monitoring, is challenging, especially across broad geographic extents that are generally remote. However, as this species congregates at small waterholes to drink daily, detection of Gouldian finch presence by aquatic eDNA sampling may be an alternative, cost-effective survey technique. The test we have developed is a useful tool that will contribute to future assessment of the distribution of this species, and one which may be elaborated into a robust population monitoring method at important locations for the species, such as the Yinberrie Hills.

Molecular methods for eDNA analysis have been changing constantly for the past decade (Jarman et al. 2018). The design of our qPCR assay for Gouldian finch eDNA involved multiple channels of fluorescent detection (Ahrberg & Neužil 2015) to provide a versatile test for the Gouldian finch and other finches. The group-specific approach for PCR amplification also lowers the chance of encountering a Gouldian finch with sequence variation in the PCR primer-binding sites leading to undetectable false negative outcomes. The test provides an opportunity to determine the presence of other finch species either by sequencing the PCR products, or by designing alternative hydrolysis probes for detecting them. The system also allows for a true internal positive control to be incorporated in the form of synthetic spike-in DNA and a hydrolysis probe for detecting it. This allows differentiation among true and false negative and positive results (Furlan & Gleeson 2017).

Aviary trial results provided the first indication that this test could detect finch and specifically Gouldian finch DNA in water which the birds had accessed — a testament to its sensitivity and potential value for detecting terrestrial species in the wild. The volumes of water tested were small because the Gouldian finch generally drinks from small pools of surface water, so any field application of this method should be capable of identifying the birds where only small quantities of water are available. Detection was successful in as little as 20 ml, which was very encouraging. The positive association between frequency of visits to the water trays and positive test results is expected, because at each visit, more eDNA is shed (Williams et al. 2018). This result suggests there is potential to explore quantitative approaches to eDNA sampling in the future.

Although the presence of more birds was associated with a greater accumulation of DNA and greater persistence, turbidity and temperature could act as counterbalances. Addition of fine sediment negatively impacted finch DNA detection without impacting control amplifications. A variety of possible reasons for this exist, but we were unable to explain this result within the scope of this study. Adsorption of eDNA to fine soil particles confounds DNA extraction efficiency (Saeki & Sakai 2009, Yu et al. 2013) and will need to be resolved in further optimisation studies (Williams et al. 2017).

The aviary trials also demonstrated specificity for finch and Gouldian finch DNA, and other species-specific eDNA assays that have taken a similar qPCR approach also reported high specificity (Thomsen et al. 2012, Takahara et al. 2013, Wilcox et al. 2013). A number of factors could influence the reliability of the eDNA tests, one of which is DNA persistence. Results from the aviary experiments indicated that with only 2 birds, the first detection could be as short as 6 h and the signal persisted for 12 h, though less when water was left in the sun. However, with as many as 55 Gouldian finches, the DNA signal could persist for up to 144 h in the sun.

The eDNA tests we have developed for finches and Gouldian finch were successful when trialled in the field, using DNA extracted from as little as 200 ml of water. The eDNA test for finches and the Gouldian finch-specific test were positive for waterholes where Gouldian and other finch species were observed each morning over 3 d. Importantly, where no Gouldian finches were observed for up to 72 h prior to water sampling, the test was negative. The species-specificity of the Gouldian finch test developed in the lab was successful in the field and consistent with the aviary validation. Where other species of finch but no Gouldian finches were observed and counted, the finch test was positive, but the Gouldian finch test was negative. These results are very promising, considering the early stages of research on the use of eDNA for vertebrate detection in the field (Ushio et al. 2017, 2018). Although the test has high specificity and there were no false positive results, there were negative results for waterholes where Gouldian finches were present, indicating that the reliability and robustness of the tests still need optimisation.

In the waterhole trials, 1 site with 60 Gouldian finches recorded on the first day of surveys was negative for water collected on Day 3 after 2 d of no Gouldian finch visits, suggesting that persistence in the field environment may be shorter than that recorded in the aviaries. Further waterhole trials are required to derive average persistence, predicated on a set of environmental conditions, which will help inform the design and interpretation of monitoring surveys that use eDNA.

Waterholes provide a completely uncontrolled environment, very different from clean drinking water in aviaries. Natural waterholes contain enzymes that break down DNA and are exposed to sun which degrades DNA. They also contain algae and other bacteria which may break down, mask or promote (by providing a surface to adhere to) eDNA signals. While it may not be possible to control or determine the direct impact of these variables, it is important to clarify and at least attempt to increase the probability of detection to an acceptable level for each survey purpose. The aviary studies also showed that fine sediment will negatively affect DNA recovery. In the waterholes tested, the turbidity was low except at one of the control sites, so the impacts of turbidity in real field situations remains unknown. The volume of water in each waterhole is also likely to influence the probability of detection. At larger waterholes where numbers are below 100 individual Gouldian finches, collecting larger volumes and/or more samples may improve the detection probability.

Resources required for biodiversity surveys include labour, time, equipment, expertise and sample handling. eDNA sampling for target species may require as little as 200 ml of water, and the approach we took cost approximately AUD 30 sample^{-1} for processing or less with bulk samples (once the test developmental work had been done). As an alternative to waterhole surveys by direct observation, eDNA sampling is relatively quick, and can be done at any time of day; it is not confined to the short periods where birds come in to drink. This potentially allows a much larger

number of waterholes to be systematically surveyed across the species' range.

In our study, water samples were filtered for DNA almost immediately, but this would be impractical if sampling across an extensive, remote area. Keeping samples cool and/or sent to a laboratory for DNA extraction within 72 h of collection is also not likely to be realistic. When we tested the effect of storing water for 14 d either cool or at room temperature, we found that the test results for the waterholes that gave high positive rates in the original test were still positive. Where there were low detection rates in the original test, the stored samples also gave low rates, albeit slightly better if stored cool. The negative controls where no birds were observed in the previous 72 h gave an occasional positive in the stored samples, which suggests that at very low concentrations the finch test can be positive, possibly reflecting residual DNA from previous visits by birds. This is not necessarily related to storage and is not likely to occur with the Gouldian finch test because of the internal DNA probe which provides both specificity and robustness. Our specific recommendations for routine eDNA detection of finches and Gouldian finches are to sample 200 ml of water, preferably at observed drinking sites within 24 h of bird sighting. Where filtering on site is not feasible, water sample collection and cool storage for up to 14 d prior to lab processing is acceptable. Based on our results from the 'real-world' waterhole trials, a positive detection indicates that Gouldian finches have visited the area at least 48 h prior to water sampling. We recognise that the test may underestimate Gouldian finch absence/presence but we are confident that a correctly executed test will not give a false positive. An ongoing waterhole campaign is currently underway to refine some of these recommendations.

For this approach to be accepted as a successful adjunct or replacement survey method for the Gouldian finch, the technology and sampling protocols need to be effectively communicated to a range of research users. Once detection probability parameters are established, the next stage is to work in collaboration with research users to undertake coordinated waterhole sampling across the species' range using a standardised, robust sampling strategy. The approach must take into account the need to measure physical and physicochemical variables, including waterhole volume, temperature and turbidity. Broad-scale systematic monitoring is essential to provide reliable data to inform the conservation status of the Gouldian finch (Bolton et al. 2016) and the response of the species to land management activities, particularly fire.

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Appendix. PERMANOVA analyses

Table A1. PERMANOVA analyses for volume, first detection and persistence trials for detection of Gouldian finch eDNA. Perms: permutations; NS: not significant

Data	Source	df	Pseudo-*F*	Unique perms	p
Minimum sample volume	Aviary	2	7	971	0.004
	Volume	3	5.7	973	0.005
	Aviary × volume	6	4.3	999	0.008
First detection	Visits	1	36.4	801	0.001
	Aviary	2	11.4	999	0.001
	Sample round	6	3.3	999	0.006
	Aviary × sample round	11	2.6	999	0.017
Persistence	Temperature	1	13.15	347	0.001
55 Gouldian finches	Aviary	5	5.73	999	0.001
Positive detection	Sample round	6	5.71	999	0.001
	Aviary × sample round	26	1.93	998	0.008
Persistence	Treatment	1	1.1	943	0.311 (NS)
Two Gouldian finchs	Sample round	3	57.6	998	0.001
Positive detection	Treatment × sample round	3	0.4	998	0.808 (NS)

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Appendix 2

eDNA pipeline

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Contents

1 Introduction

eDNA is a bioinformatic pipeline facilitating the analysis of Amplicon Sequence Variants from environmental DNA samples based on the *DADA2* pipeline [1]. It is capable of processing sequencing data from multiplex PCR assays with unser-defined primer sets. The pipeline is implemented in Microsoft R Open v3.5.3, with third-party R packages and programs as dependencies.

Pipeline scripts, dependencies, and the R runtime environment are provided as a Docker image based on Ubuntu 20.04. This documentation assumes that the pipeline is installed and used on a computer running the latest Windows 10 operating system. It should also be possible (but has not been explicitly tested) to use the pipeline Docker image on macOS or other Linux/Unix distros with minor modifications.

By running this pipeline you accept the Microsoft R Open and Math Kernel Libraries license terms.

2 Change log

- Version 1.0: Initial version.

3 Prerequisites

1. **Hardware requirements**

- $\geq$ 5 GiB free disc space
- $\geq$ 10 GiB free RAM

2. **Docker**
For installation on Windows 10, follow the instructions below:

- Install Docker Desktop for Windows
- Install Windows Subsystem for Linux (WSL) 2
It is recommended to use Ubuntu 20.04 LTS as your WSL Linux distro.

4 Installation

1. Unpack the archive file *eDNA_pipeline.zip* you received by email on your hard disk (let's say, on partition `C:\`; if you prefer a different partition, change the letter accordingly). This will create a folder `C:\eDNA_pipeline` with the necessary folder structure and files.
2. Make sure Docker Desktop is running.
3. Open WSL.
4. Create a link to the pipeline folder in your home folder:
`$ ln -s /mnt/c/eDNA_pipeline .`
5. Create a link of the pipeline execution script in the local binaries folder:
`$ sudo cp -s ~/eDNA_pipeline/eDNA /usr/local/bin`
6. Pull the Docker image:
`$ docker pull omics2view/eDNA:1.0`

5 Running the pipeline

1. Open WSL.
2. Navigate to the pipeline folder:
`$ cd eDNA_pipeline`
3. Example calls:
   - Default settings (analysis mode, all tasks, all primer pairs):
   `$ eDNA` (requires fastq input and reference files to be in their respective folders)
   - Explicit operation mode (e.g., database construction) running all tasks:
   `$ eDNA --mode db`
   - Database construction for explicit primer pairs (use double quotes for more than one primer):
   `$ eDNA --mode db --primers "Euk Mamm02"`
   - Execute explicit tasks (e.g., getting reference sequences and taxonomy; use double quotes for more than one task):
   `$ eDNA --mode db --tasks "get_sequences get_taxonomy"`
   - Overview of command line arguments:
   `$ eDNA --help`

For detailed information on available options, please see the following sections.

6 Pipeline components

6.1 Folder structure

6.1.1 Folder *data*

The *data* folder contains reference database files specific to each primer pair (in subfolder *reference*). It also contains the configuration file *config.json*.

The contents of the *reference* subfolder should not be edited by the user.

6.1.2 Folder *input*

This is where the user places input files.

- Subfolder *fastq* holds paired read data files. Their naming *must* adhere to the pattern `X_S[0-9]_L[0-9]_R[12]_001.fastq.gz`, where `X` can be a sample ID of convenient length which may contain characters `A-Z`, `a-z`, `0-9`, and `.-_` .
- Subfolder *reference* holds reference input files:
  - You can obtain reference data for a gene you have amplified by submitting a query to NCBI (e.g., this query for the mitochondrial 12S rRNA in birds).
    Examples for queries:
    * `12S[All fields] AND gene_in_mitochondrion[PROP] AND Aves[Organism]`
    * `16S[All fields] AND gene_in_mitochondrion[PROP] AND Mammals[Organism] NOT "Homo sapiens"[Organism]`
    * `18S[All fields] AND gene_in_genomic[PROP] AND Vertebrates[Organism] NOT "Homo sapiens"[Organism]`

    Download the result as a list of accession numbers in simple *text* format (one entry per line) using the `Send to:` dialogue. The file must be named after the primer pair it references (e.g., *Aves02.txt*).
  - You can also provide reference sequences in multi-fasta format, named after the primer pair it references (e.g., *Euk.fasta*). Each fasta header must contain a *unique* reference ID (like NCBI's accession numbers), followed by an NCBI taxonomy ID:

    ```
    >TDNA_2_CDU_eDNA_B1_Euk NCBI:txid9315
    ACATCCGGATACGGGATCCCGTAGACTAGC...
    ```

    The fasta sequence *must not* be flanked by primer sequences.

6.1.3 Folder *log*

This folder contains the pipeline's log and information files:

- File *eDNA.log* captures the content of the terminal window.
- File *sort_clean.log* provides the diagnostic output produced in analysis task `sort_clean`.
- File *sessionInfo.txt* provides detailed information about the version of R, the operating system, and the R packages used by the pipeline.

6.1.4 Folder *output*

When the pipeline is run in analysis mode, a subfolder *process* is created that contains the output of the tasks executed in the analysis process. This enables the user to inspect intermediate results. Upon completion of the analysis, a subfolder with result files is created for each selected primer pair.

6.2 File *eDNA*

The Linux shell script *eDNA* provides a command wrapper for convenient execution of the pipeline. It runs the Docker image with correct parameters, connects the folder structure to its counterpart within the Docker

container, and logs the progress.

The contents of the *eDNA* file should not be edited by the user.

6.3 File *config.json*

Important parameters of the pipeline, which rarely need to be changed, are stored in the configuration file *config.json*:

- `"primers"`: List of primer pairs with name, forward primer sequence, and reverse primer sequence
- `"targets"`: List of priming targets with primer pair name and gene designation as used by NCBI
- `"expectedLengthRanges"`: List of amplicon length ranges with primer pair name, expected minimum length, and expected maximum length
- `"randomSeed"`: Integer value passed to R's random number generator; used to get identical results when running the pipeline repeatedly with the same input
- `"mismatch"`: Number of mismatches (substitutions) allowed between primer and target sequence (`0-3`)
- `"chunkSize"`: Number of NCBI database entries to be downloaded simultaneously (`1-500`)
- `"maxExpectedErrors"`: Maximum number of expected errors for forward and reverse reads
- `"minOverlap"`: Minimum number of base pairs in the overlap region of forward and reverse read to form a contig
- `"confidenceThreshold"`: Minimum confidence threshold for taxonomic classification (`0-100`)

7 General concepts

7.1 Operation modes

The eDNA pipeline has two main operation modes, *database* and *analysis*. They are invoked by `$ eDNA --mode db` and `$ eDNA --mode analysis`, respectively. As *analysis* is the default mode, you can also omit parameter `--mode analysis` for this mode.

7.2 Reference database

Taxonomic classification of Amplicon Sequence Variants is facilitated by an internal database. It contains reference sequences and taxonomic information specific to each primer pair declared in the configuration file *config.json*. This information is used to train the *IDTAXA Classifier* of the *DECIPHER* package [3]. For each primer pair used in *analysis* mode, a corresponding database trainset must be available for the *eDNA* pipeline to work.

An existing database can be extended by providing new accession numbers or fasta sequences. Reference sequence accession numbers / reference IDs that have already been processed are ignored when resubmitted to the pipeline. Database entries corresponding to a specific primer pair can be deleted with command line parameter `--clearreference`, followed by the name of the primer pair, e.g., `$ eDNA --mode db --clearreference Aves02`. To declare more than one primer pair, write their names consecutively (separated by a space) in double quotes, e.g., `$ eDNA --mode db --clearreference "Aves02 Mamm02"`. The pipeline then deletes the selected databases and rebuilds them from scratch.

Database entries corresponding to a specific primer pair *must* be rebuilt (using command line parameter `--clearreference` as described above) if any of the following parameters in the configuration file *config.json* have been changed:

- `"primers"`
- `"targets"`
- `"expectedLengthRanges"`
- `"randomSeed"`
- `"mismatch"`

7.3 Tasks

Each operation mode comprises several tasks, which are executed one after the other in a fixed order. This modular structure allows the user to interrupt or repeat individual processes without having to restart the entire pipeline. Tasks are declared with command line parameter `--tasks`, followed by the task name, e.g., `$ eDNA --mode db --tasks stats`. Several tasks can be declared by writing their names consecutively (separated by a space) in double quotes, e.g., `$ eDNA --mode db --tasks "get_sequences get_taxonomy"`. Omitting `--tasks` or declaring `--tasks all` runs all tasks defined for the respective operation mode.

7.4 Primers

The pipeline is designed to process sequencing data from multiplex PCR assays. By default, it will run the declared tasks for each PCR primer pair described in the configuration file *config.json*. Tasks can be restricted to specific primer pairs with the command line parameter `--primers`, followed by the primer pair name. To declare more than one primer pair, write their names consecutively (separated by a space) in double quotes, e.g., `$ eDNA --mode db --primers "Aves02 Mamm02"`.

7.5 Session info

Command line argument `--sessioninfo` triggers the export of R session info to file *sessionInfo.txt* in the *log folder*. It can be set regardless of other arguments.

8 Description of tasks

8.1 Database mode

8.1.1 Task `get_sequences`

If the user provided a list of NCBI accession numbers in the *input* folder, the task retrieves their sequences from the NCBI nucleotide database. This may take several minutes depending on the length of the input list, the bandwidth of the internet connection to the NCBI server, and the server load. If the user provided a fasta file with reference sequences, the task simply imports them.

Accession lists and fasta files must be submitted in separate calls to `$ eDNA --mode db`.

8.1.2 Task `get_taxonomy`

This task retrieves taxonomic information for each reference sequence from the NCBI taxonomy database. This may take several minutes depending on the number of entries, the bandwidth of the internet connection to the NCBI server, and the server load. The task extracts entries at main taxonomic levels and stores them together with the reference sequences in a tabular format.

8.1.3 Task `extract_amplicons`

"*In-silico* amplicon sequences" corresponding to the target region of a corresponding primer pair are extracted from sequences retrieved from NCBI. This is done by determining the priming regions in the reference sequence followed by excising the sequence part between these regions. If no primer matches are found, the process is repeated with the reverse-complement of the reference sequence. The length of the excised amplicons must lie within the expected length range defined for the corresponding primer pair in the configuration file *config.json*. The process may take several minutes depending on the number reference sequences and the number of logical processors available to the pipeline.

References provided in fasta format are assumed to be amplicon sequences *without* flanking primers.

8.1.4 Task `train`

Reference amplicon sequences and taxonomic information are used to train the *IDTAXA Classifier* of the *DECIPHER* package [3]. Only unique amplicon-taxonomy combinations are used for training.

8.1.5 Task `stats`

This task displays summary statistics for the database entries of each primer pair.

8.2 Analysis mode

8.2.1 Task `sort_clean`

The task uses *BBMap* [2] to look for primer sequences defined in the configuration file *config.json* in the input fastq files. If the 5' ends of both the forward and the reverse read start with a known forward and reverse primer sequence, respectively, the read pair is held back. The maximum allowed number of mismatches (substitutions) between read and primer sequence is taken from the `"mismatch"` parameter in the configuration file.

Subsequently, primer and adapter residues at their 3' ends are removed from the reads. The sorted and cleaned read pairs are stored in paired fastq files in *eDNA_pipeline/output/process/clean*, with one file pair for each sample ID and primer pair.

Read pairs that failed matching to a known primer pair go to paired fastq files in *eDNA_pipeline/output/process/nomatch*. If the number of sorted and cleaned read pairs for a particular primer pair does not meet expectations, it is worth examining these files to see if anything went wrong at this stage.

8.2.2 Task `filter`

The task applies the "filter and trim" process of the *DADA2* pipeline successively to the cleaned reads of each selected primer pair. In particular, it uses the `"expectedLengthRanges"` and `"minOverlap"` parameters in the configuration file *config.json* to determine the minimum read length. It further takes the value of the `"maxExpectedErrors"` parameter in the configuration file as the maximum number of "expected errors" allowed in a read.

8.2.3 Task `learn_error_rates`

The task applies the "learn error rates" process of the *DADA2* pipeline successively to the filtered reads of each selected primer pair.

8.2.4 Task `composition_inference`

The task executes the core sample inference algorithm of the *DADA2* pipeline for denoising forward and reverse reads. The algorithm is successively applied to the filtered reads of each selected primer pair.

8.2.5 Task `merge_pairs`

Full amplicon sequences are obtained by aligning the denoised forward reads with the reverse-complement of the corresponding denoised reverse reads, and then constructing the merged "contig" sequences. The latter are referred to Amplicon Sequence Variants (ASVs). The procedure is successively applied to denoised reads of each selected primer pair.

8.2.6 Task `sequence_table`

In this task, a table of ASV counts per sample is successively created for each selected primer pair. It also applies the `"expectedLengthRanges"` parameter in the configuration file *config.json* to remove ASVs of atypical length from the respective count table.

8.2.7 Task `remove_chimeras`

While substitution and inDel errors are corrected in the preceding tasks, chimeras are still present in the data at this stage. Sequences are chimeric if they can be exactly reconstructed by combining a *left* and a *right* segment from *two* more abundant "parent" sequences. This is why they are coined "*bi*meras" in the *DADA2* pipeline. The procedure successively removes chimeric ASVs from the count table of each selected primer pair.

8.2.8 Task `assign_taxonomy`

The primer-specific trainset produced by the *IDTAXA Classifier* of the *DECIPHER* package [3] is used to taxonomically classify ASVs. The task applies the `"confidenceThreshold"` parameter in the configuration file *config.json* to filter out ASVs with unreliable classifications.

8.2.9 Task `phylogeny`

If at least three ASVs with valid taxonomic classification are present for a selected primer pair, the task aligns these sequences and infers their phylogeny from this alignment. Phylogenetic calculations are based on the Neighbor-Joining method [4].

Phylogenetic tree calculation intrinsically assumes that the ASVs in question are related. This means that they can be aligned in a non-random way and have finite pairwise dissimilarity values derived from that alignment. The presence of infinite pairwise dissimilarity values indicates the presence of incompatible ASVs, e.g., through unspecific priming. In that case, the task issues a warning that the resulting phylogeny will be biased. Although a biased phylogeny should not be used for community analysis, it may still serve a diagnostic purpose to identify abnormal ASVs in the data.

8.2.10 Task `stats`

The task produces and displays summary statistics on the number of read pairs and ASVs, respectively, that made it through each of the aforementioned analysis tasks.

8.2.11 Task `output`

Upon completion of the analysis, a subfolder with result files for each selected primer pair is created in the *output* folder. Each subfolder contains up to six result files, with the name of the primer pair as prefix (expressed as wildcard asterisk * in the list below):

- File **_stats.csv* contains the read pair / ASV count statistics.
- File **_features.csv* contains ASV names (md5 checksums of ASV sequences) and sequences.
- File **_counts.csv* contains ASV names and per-sample counts.
- File **_taxonomy.csv* contains ASV names, taxonomic information, and confidence values.
- File **_phylogeny.tree* contains the inferred ASV phylogeny in Newick notation with ASV names as tip labels.
- File **_phyloseq.rds* contains a *phyloseq* [5] object that combines ASV counts, sequences, taxonomy, and phylogeny, and can be imported into R with `readRDS()`.

9 References

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Appendix 3

Table 1: Sample IDs.

Accession #	Site name	Water sample ID	General location
eDNA1	North 2	neg	Wollogorang
eDNA2	North 2	Sample 1	
eDNA3	North 2	Sample 2	
eDNA4	North 2	Sample 3	
eDNA5	North 2	Sample 4	
eDNA6	North 2	Sample 5	
eDNA7	North 2	Sample 6	
eDNA8	Camel Creek 3	neg	
eDNA9	Camel Creek 3	Sample 1	
eDNA10	Camel Creek 3	Sample 2	
eDNA11	Camel Creek 3	Sample 3	
eDNA12	Camel Creek 3	Sample 4	
eDNA13	Camel Creek 3	Sample 5	
eDNA14	Camel Creek 3	Sample 6	
eDNA15	Camel Creek	neg	
eDNA16	Camel Creek	Sample 1	
eDNA17	Camel Creek	Sample 2	
eDNA18	Camel Creek	Sample 3	
eDNA19	Camel Creek	Sample 4	
eDNA20	Camel Creek	Sample 5	
eDNA21	Camel Creek	Sample 6	
eDNA22	North 1	neg	
eDNA23	North 1	Sample 1	
eDNA24	North 1	Sample 2	
eDNA25	North 1	Sample 3	
eDNA26	North 1	Sample 4	
eDNA27	North 1	Sample 5	
eDNA28	North 1	Sample 6	
eDNA29	Banyan 5	neg	
eDNA30	Banyan 5	Sample 1	
eDNA31	Banyan 5	Sample 2	
eDNA32	Banyan 5	Sample 3	
eDNA33	Banyan 5	Sample 4	
eDNA34	Banyan 5	Sample 5	
eDNA35	Banyan 5	Sample 6	
eDNA36	Redbank 3	neg	
eDNA37	Redbank 3	Sample 1	
eDNA38	Redbank 3	Sample 2	
eDNA39	Redbank 3	Sample 3	
eDNA40	Redbank 3	Sample 4	
eDNA41	Redbank 3	Sample 5	
eDNA42	Redbank 3	Sample 6	

eDNA43	Rocky Creek 1	neg	
eDNA44	Rocky Creek 1	Sample 1	
eDNA45	Rocky Creek 1	Sample 2	
eDNA46	Rocky Creek 1	Sample 3	
eDNA47	Rocky Creek 1	Sample 4	
eDNA48	Rocky Creek 1	Sample 5	
eDNA49	Rocky Creek 1	Sample 6	
eDNA50	Banyan Gorge	neg	
eDNA51	Banyan Gorge	Sample 1	
eDNA52	Banyan Gorge	Sample 2	
eDNA53	Banyan Gorge	Sample 3	
eDNA54	Banyan Gorge	Sample 4	
eDNA55	Banyan Gorge	Sample 5	
eDNA56	Banyan Gorge	Sample 6	
eDNA57	Aquarium 1	neg	
eDNA58	Aquarium 1	Sample 1	
eDNA59	Aquarium 1	Sample 2	
eDNA60	Aquarium 1	Sample 3	
eDNA61	Aquarium 1	Sample 4	
eDNA62	Aquarium 1	Sample 5	
eDNA63	Aquarium 1	Sample 6	
eDNA64	Moonlight 1	neg	
eDNA65	Moonlight 1	Sample 1	
eDNA66	Moonlight 1	Sample 2	
eDNA67	Moonlight 1	Sample 3	
eDNA68	Moonlight 1	Sample 4	
eDNA69	Moonlight 1	Sample 5	
eDNA70	Moonlight 1	Sample 6	
eDNA71	McDermott 3	neg	
eDNA72	McDermott 3	Sample 1	
eDNA73	McDermott 3	Sample 2	
eDNA74	McDermott 3	Sample 3	
eDNA75	McDermott 3	Sample 4	
eDNA76	McDermott 3	Sample 5	
eDNA77	McDermott 3	Sample 6	
eDNA78	McDermott 2	neg	
eDNA79	McDermott 2	Sample 1	
eDNA80	McDermott 2	Sample 2	
eDNA81	McDermott 2	Sample 3	
eDNA82	McDermott 2	Sample 4	
eDNA83	McDermott 2	Sample 5	
eDNA84	McDermott 2	Sample 6	
eDNA157	Joker	Neg	Arid Zone
eDNA158	Joker	Sample 1	
eDNA159	Joker	Sample 2	
eDNA160	Joker	Sample 3	
eDNA161	SIMG	Neg	

eDNA162	SIMG	Sample 1	
eDNA163	SIMG	Sample 2	
eDNA164	SIMG	Sample 3	
eDNA165	TGJH	Neg	
eDNA166	TGJH	Sample 1	
eDNA167	TGJH	Sample 2	
eDNA168	TGJH	Sample 3	
eDNA169	OrmG	Neg	
eDNA170	OrmG	Sample 1	
eDNA171	OrmG	Sample 2	
eDNA172	OrmG	Sample 3	
eDNA173	GS	Neg	
eDNA174	GS	Sample 1	
eDNA175	GS	Sample 3	
eDNA176	RBG	Neg	
eDNA177	RBG	Sample 1	
eDNA178	RBG	Sample 2	
eDNA179	RBG	Sample 3	
eDNA180	RBG2	Neg	
eDNA181	RBG2	Sample 1	
eDNA182	RBG2	Sample 2	
eDNA183	RBG2	Sample 3	
eDNA184	GY	Neg	
eDNA185	GY	Sample 1	
eDNA186	GY	Sample 2	
eDNA187	GY	Sample 3	
eDNA188	RGHR	Neg	
eDNA189	RGHR	Sample 1	
eDNA190	RGHR	Sample 2	
eDNA191	RGHR	Sample 3	
eDNA192	R2B1	Neg	
eDNA193	R2B1	Sample 1	
eDNA194	R2B1	Sample 2	
eDNA195	R2B1	Sample 3	
eDNA196	PORT	Neg	
eDNA197	PORT	Sample 1	
eDNA198	PORT	Sample 2	
eDNA199	PORT	Sample 3	
eDNA200	ECK	Neg	
eDNA201	ECK	Sample 1	
eDNA202	ECK	Sample 2	
eDNA203	ECK	Sample 3	
eDNA204	BG	Neg	
eDNA205	BG	Sample 1	
eDNA206	BG	Sample 2	
eDNA207	BG	Sample 3	
eDNA208	SPG	Neg	

eDNA209	SPG	Sample 1	
eDNA210	SPG	Sample 2	
eDNA211	SPG	Sample 3	
eDNA212	TG	Neg	
eDNA213	TG	Sample 1	
eDNA214	TG	Sample 2	
eDNA215	TG	Sample 3	
eDNA216	HG	Neg	
eDNA217	HG	Sample 1	
eDNA218	HG	Sample 2	
eDNA219	HG	Sample 3	
eDNA220	RG	Neg	
eDNA221	RG	Sample 1	
eDNA222	RG	Sample 2	
eDNA223	RG	Sample 3	
eDNA224	RG2	Neg	
eDNA225	RG2	Sample 1	
eDNA226	RG2	Sample 2	
eDNA227	RG2	Sample 3	
eDNA228	BWCK	Neg	
eDNA229	BWCK	Sample 1	
eDNA230	BWCK	Sample 2	
eDNA231	BWCK	Sample 3	
eDNA232	ARLMINE	Neg	
eDNA233	ARLMINE	Sample 1	
eDNA234	ARLMINE	Sample 2	
eDNA235	ARLMINE	Sample 3	
eDNA236	FH	Neg	
eDNA237	FH	Sample 1	
eDNA238	FH	Sample 2	
eDNA239	FH	Sample 3	
eDNA240	JOKER2	Neg	
eDNA241	JOKER2	Sample 1	
eDNA242	JOKER2	Sample 2	
eDNA243	JOKER2	Sample 3	
eDNA244	RG3	Neg	
eDNA245	RG3	Sample 1	
eDNA246	RG3	Sample 2	
eDNA247	RG3	Sample 3	
eDNA248	RZB2	Neg	
eDNA249	RZB2	Sample 1	
eDNA250	RZB2	Sample 2	
eDNA251	RZB2	Sample 3	

Table 2: Mammal02_counts.

ASV	eDNA-1	eDNA-10	eDNA-11	eDNA-12	eDNA-13	eDNA-14	eDNA-15	eDNA-157	eDNA-158	eDNA-159
0bd8aa353000d7200eab44f0f7f75d90										
6a7be99afeee124f7a76f2998ebb597e										
7667427bcd6b201b6d6c106497acef37										
2bb407c368414e0969ccc76edf6c5c9d										
880459d1858aa106016e9cdd8c6e73e9	4				19	20	10			
59bcd49cb2e198f72833788401dcc7cb										
fc0f98f44f1bbe3177c059ae333e5c0f	5				18	16	7			
cc8940197219ad6ac1ce5759fe646fc1						1	1			
10d58e3b59f907c90e9dab491d2aae27										
bf51cc6e1dd2c7fde081c04a216c0172						1				
2c34cf16fe43896b9c58cc3bbc37f8e3										
66b1a8b2bc6d0b164e16ea88b1ec91bd										
9245b027bf154b1c4c9594ae843b26cf										
8d77fb3cc5c248e3376a9c0df5386bdf	9				21	14	15			
f83f71f93f6e908cb4f8cabd09a24cf8					3					
81a08c4056698cb10741d61e751704cc										
f691c9f1b907b4d8a601e493a5a358b2	2				7	3	1			
8f2fb4e5b8ffc59f7a1b7c1b995e25f4										
07f648cadd099e3c0494fc6550aabdca										
2ef7d952c54b153e64397fa47c24abff										
b87af5c73c103467cba30de2efed1929										
8f7dd9f608c87dd624f0d2429b923619										
fc5372f0ed9dde409b5b89f572c30840										
c63c60d9b4ab2d48cafd0786362c0a3c										
5c89d7b233dc5bc566aea8d8523050a8										
a961095895dc52b6e3810dbcc1fb9f91										
b331712b0c56a69a7fa95aa34017cfbd										
f625435363c3185d62cfad84d4b132e3										

ASV	eDNA-1	eDNA-10	eDNA-11	eDNA-12	eDNA-13	eDNA-14	eDNA-15	eDNA-157	eDNA-158	eDNA-159
794f0e4b71828ccdc76300b512ef2d7d	1				2	3				
4937cc30738dbb33464513afdebc8bcf										
b11e48466c55159b83352aaa222bc07e					1	1	2			
c09f21dfe23cf8b1f222672305d38944										
b1c7e99bfa4a11334f2497d033d655bc										
78cc4d1a6e5a85ca641eda16a7fc78c9										
5d5142f1ec3aeed0c0da48a125934182										
c253a68c33b91180ff20d271fdac80ab										
456fb56e167a55e0a5783f279fad02ee										
6c40adf1ea6f603a98d4212378aa6ca6										

ASV	eDNA-16	eDNA-160	eDNA-161	eDNA-162	eDNA-163	eDNA-164	eDNA-165	eDNA-166	eDNA-167	eDNA-168	eDNA-169
0bd8aa353000d7200eab44f0f7f75d90											
6a7be99afeee124f7a76f2998ebb597e											
7667427bcd6b201b6d6c106497acef37											
2bb407c368414e0969ccc76edf6c5c9d											
880459d1858aa106016e9cdd8c6e73e9	11					22					
59bcd49cb2e198f72833788401dcc7cb											
fc0f98f44f1bbe3177c059ae333e5c0f	7					12					
cc8940197219ad6ac1ce5759fe646fc1						1					
10d58e3b59f907c90e9dab491d2aae27											
bf51cc6e1dd2c7fde081c04a216c0172	1										
2c34cf16fe43896b9c58cc3bbc37f8e3											
66b1a8b2bc6d0b164e16ea88b1ec91bd											
9245b027bf154b1c4c9594ae843b26cf											
8d77fb3cc5c248e3376a9c0df5386bdf	8					19					
f83f71f93f6e908cb4f8cabd09a24cf8											
81a08c4056698cb10741d61e751704cc											
f691c9f1b907b4d8a601e493a5a358b2	1					4					
8f2fb4e5b8ffc59f7a1b7c1b995e25f4											

ASV	eDNA-16	eDNA-160	eDNA-161	eDNA-162	eDNA-163	eDNA-164	eDNA-165	eDNA-166	eDNA-167	eDNA-168	eDNA-169
07f648cadd099e3c0494fc6550aabdca											
2ef7d952c54b153e64397fa47c24abff											
b87af5c73c103467cba30de2efed1929											
8f7dd9f608c87dd624f0d2429b923619											
fc5372f0ed9dde409b5b89f572c30840											
c63c60d9b4ab2d48cafd0786362c0a3c											
5c89d7b233dc5bc566aea8d8523050a8											
a961095895dc52b6e3810dbcc1fb9f91											
b331712b0c56a69a7fa95aa34017cfbd											
f625435363c3185d62cfad84d4b132e3											
794f0e4b71828ccdc76300b512ef2d7d											
4937cc30738dbb33464513afdebc8bcf											
b11e48466c55159b83352aaa222bc07e		2									
c09f21dfe23cf8b1f222672305d38944											
b1c7e99bfa4a11334f2497d033d655bc											
78cc4d1a6e5a85ca641eda16a7fc78c9											
5d5142f1ec3aeed0c0da48a125934182											
c253a68c33b91180ff20d271fdac80ab											
456fb56e167a55e0a5783f279fad02ee											
6c40adf1ea6f603a98d4212378aa6ca6											

ASV	eDNA-17	eDNA-170	eDNA-171	eDNA-172	eDNA-173	eDNA-174	eDNA-175	eDNA-176	eDNA-177	eDNA-178	eDNA-179
0bd8aa353000d7200eab44f0f7f75d90											
6a7be99afeee124f7a76f2998ebb597e											
7667427bcd6b201b6d6c106497acef37											
2bb407c368414e0969ccc76edf6c5c9d											
880459d1858aa106016e9cdd8c6e73e9		6			9						
59bcd49cb2e198f72833788401dcc7cb											
fc0f98f44f1bbe3177c059ae333e5c0f		9			6						
cc8940197219ad6ac1ce5759fe646fc1											

ASV	eDNA-17	eDNA-170	eDNA-171	eDNA-172	eDNA-173	eDNA-174	eDNA-175	eDNA-176	eDNA-177	eDNA-178	eDNA-179
10d58e3b59f907c90e9dab491d2aae27											
bf51cc6e1dd2c7fde081c04a216c0172	78										
2c34cf16fe43896b9c58cc3bbc37f8e3											
66b1a8b2bc6d0b164e16ea88b1ec91bd											
9245b027bf154b1c4c9594ae843b26cf											
8d77fb3cc5c248e3376a9c0df5386bdf	14			15							
f83f71f93f6e908cb4f8cabd09a24cf8											
81a08c4056698cb10741d61e751704cc											
f691c9f1b907b4d8a601e493a5a358b2	1										
8f2fb4e5b8ffc59f7a1b7c1b995e25f4											
07f648cadd099e3c0494fc6550aabdca											
2ef7d952c54b153e64397fa47c24abff				2							
b87af5c73c103467cba30de2efed1929											
8f7dd9f608c87dd624f0d2429b923619											
fc5372f0ed9dde409b5b89f572c30840											
c63c60d9b4ab2d48cafd0786362c0a3c											
5c89d7b233dc5bc566aea8d8523050a8											
a961095895dc52b6e3810dbcc1fb9f91											
b331712b0c56a69a7fa95aa34017cfbd											
f625435363c3185d62cfad84d4b132e3											
794f0e4b71828ccdc76300b512ef2d7d				1							
4937cc30738dbb33464513afdebc8bcf											
b11e48466c55159b83352aaa222bc07e											
c09f21dfe23cf8b1f222672305d38944											
b1c7e99bfa4a11334f2497d033d655bc											
78cc4d1a6e5a85ca641eda16a7fc78c9											
5d5142f1ec3aeed0c0da48a125934182											
c253a68c33b91180ff20d271fdac80ab											
456fb56e167a55e0a5783f279fad02ee											
6c40adf1ea6f603a98d4212378aa6ca6											

ASV	eDNA-18	eDNA-180	eDNA-181	eDNA-182	eDNA-183	eDNA-184	eDNA-185	eDNA-186	eDNA-187	eDNA-188	eDNA-189
0bd8aa353000d7200eab44f0f7f75d90											
6a7be99afeee124f7a76f2998ebb597e											
7667427bcd6b201b6d6c106497acef37											
2bb407c368414e0969ccc76edf6c5c9d											
880459d1858aa106016e9cdd8c6e73e9		17								22	
59bcd49cb2e198f72833788401dcc7cb											
fc0f98f44f1bbe3177c059ae333e5c0f		11								9	
cc8940197219ad6ac1ce5759fe646fc1										1	
10d58e3b59f907c90e9dab491d2aae27											
bf51cc6e1dd2c7fde081c04a216c0172										3	
2c34cf16fe43896b9c58cc3bbc37f8e3											
66b1a8b2bc6d0b164e16ea88b1ec91bd											
9245b027bf154b1c4c9594ae843b26cf											
8d77fb3cc5c248e3376a9c0df5386bdf		16								17	
f83f71f93f6e908cb4f8cabd09a24cf8											
81a08c4056698cb10741d61e751704cc											
f691c9f1b907b4d8a601e493a5a358b2		3								5	
8f2fb4e5b8ffc59f7a1b7c1b995e25f4											
07f648cadd099e3c0494fc6550aabdca											
2ef7d952c54b153e64397fa47c24abff											
b87af5c73c103467cba30de2efed1929											
8f7dd9f608c87dd624f0d2429b923619											
fc5372f0ed9dde409b5b89f572c30840											
c63c60d9b4ab2d48cafd0786362c0a3c											
5c89d7b233dc5bc566aea8d8523050a8											
a961095895dc52b6e3810dbcc1fb9f91											
b331712b0c56a69a7fa95aa34017cfbd											
f625435363c3185d62cfad84d4b132e3											
794f0e4b71828ccdc76300b512ef2d7d											
4937cc30738dbb33464513afdebc8bcf											
b11e48466c55159b83352aaa222bc07e											

ASV	eDNA-18	eDNA-180	eDNA-181	eDNA-182	eDNA-183	eDNA-184	eDNA-185	eDNA-186	eDNA-187	eDNA-188	eDNA-189
c09f21dfe23cf8b1f222672305d38944											
b1c7e99bfa4a11334f2497d033d655bc											
78cc4d1a6e5a85ca641eda16a7fc78c9											
5d5142f1ec3aeed0c0da48a125934182											
c253a68c33b91180ff20d271fdac80ab											
456fb56e167a55e0a5783f279fad02ee											
6c40adf1ea6f603a98d4212378aa6ca6											

ASV	eDNA-19	eDNA-190	eDNA-191	eDNA-192	eDNA-193	eDNA-194	eDNA-195	eDNA-196	eDNA-197	eDNA-198	eDNA-199
0bd8aa353000d7200eab44f0f7f75d90											
6a7be99afeee124f7a76f2998ebb597e											
7667427bcd6b201b6d6c106497acef37											
2bb407c368414e0969ccc76edf6c5c9d											
880459d1858aa106016e9cdd8c6e73e9								22			
59bcd49cb2e198f72833788401dcc7cb											
fc0f98f44f1bbe3177c059ae333e5c0f								15			
cc8940197219ad6ac1ce5759fe646fc1											
10d58e3b59f907c90e9dab491d2aae27											
bf51cc6e1dd2c7fde081c04a216c0172											
2c34cf16fe43896b9c58cc3bbc37f8e3											
66b1a8b2bc6d0b164e16ea88b1ec91bd											
9245b027bf154b1c4c9594ae843b26cf											
8d77fb3cc5c248e3376a9c0df5386bdf								11			
f83f71f93f6e908cb4f8cabd09a24cf8											
81a08c4056698cb10741d61e751704cc											
f691c9f1b907b4d8a601e493a5a358b2								5			
8f2fb4e5b8ffc59f7a1b7c1b995e25f4											
07f648cadd099e3c0494fc6550aabdca											
2ef7d952c54b153e64397fa47c24abff											
b87af5c73c103467cba30de2efed1929											

ASV	eDNA-19	eDNA-190	eDNA-191	eDNA-192	eDNA-193	eDNA-194	eDNA-195	eDNA-196	eDNA-197	eDNA-198	eDNA-199
8f7dd9f608c87dd624f0d2429b923619											
fc5372f0ed9dde409b5b89f572c30840											
c63c60d9b4ab2d48cafd0786362c0a3c											
5c89d7b233dc5bc566aea8d8523050a8											
a961095895dc52b6e3810dbcc1fb9f91											
b331712b0c56a69a7fa95aa34017cfbd											
f625435363c3185d62cfad84d4b132e3											
794f0e4b71828ccdc76300b512ef2d7d											
4937cc30738dbb33464513afdebc8bcf											
b11e48466c55159b83352aaa222bc07e											
c09f21dfe23cf8b1f222672305d38944											
b1c7e99bfa4a11334f2497d033d655bc											
78cc4d1a6e5a85ca641eda16a7fc78c9											
5d5142f1ec3aeed0c0da48a125934182											
c253a68c33b91180ff20d271fdac80ab											
456fb56e167a55e0a5783f279fad02ee											
6c40adf1ea6f603a98d4212378aa6ca6											

ASV	eDNA-2	eDNA-20	eDNA-200	eDNA-201	eDNA-202	eDNA-203	eDNA-204	eDNA-205	eDNA-206	eDNA-207	eDNA-208
0bd8aa353000d7200eab44f0f7f75d90											
6a7be99afeee124f7a76f2998ebb597e											
7667427bcd6b201b6d6c106497acef37											
2bb407c368414e0969ccc76edf6c5c9d											
880459d1858aa106016e9cdd8c6e73e9							13				
59bcd49cb2e198f72833788401dcc7cb											
fc0f98f44f1bbe3177c059ae333e5c0f		1					7				
cc8940197219ad6ac1ce5759fe646fc1											
10d58e3b59f907c90e9dab491d2aae27											
bf51cc6e1dd2c7fde081c04a216c0172											
2c34cf16fe43896b9c58cc3bbc37f8e3											

ASV	eDNA-2	eDNA-20	eDNA-200	eDNA-201	eDNA-202	eDNA-203	eDNA-204	eDNA-205	eDNA-206	eDNA-207	eDNA-208
66b1a8b2bc6d0b164e16ea88b1ec91bd											
9245b027bf154b1c4c9594ae843b26cf											
8d77fb3cc5c248e3376a9c0df5386bdf							11				
f83f71f93f6e908cb4f8cabd09a24cf8											
81a08c4056698cb10741d61e751704cc											
f691c9f1b907b4d8a601e493a5a358b2											
8f2fb4e5b8ffc59f7a1b7c1b995e25f4											
07f648cadd099e3c0494fc6550aabdca											
2ef7d952c54b153e64397fa47c24abff											
b87af5c73c103467cba30de2efed1929											
8f7dd9f608c87dd624f0d2429b923619											
fc5372f0ed9dde409b5b89f572c30840											
c63c60d9b4ab2d48cafd0786362c0a3c											
5c89d7b233dc5bc566aea8d8523050a8											
a961095895dc52b6e3810dbcc1fb9f91											
b331712b0c56a69a7fa95aa34017cfbd											
f625435363c3185d62cfad84d4b132e3											
794f0e4b71828ccdc76300b512ef2d7d											
4937cc30738dbb33464513afdebc8bcf			3								
b11e48466c55159b83352aaa222bc07e											
c09f21dfe23cf8b1f222672305d38944											
b1c7e99bfa4a11334f2497d033d655bc											
78cc4d1a6e5a85ca641eda16a7fc78c9											
5d5142f1ec3aeed0c0da48a125934182											
c253a68c33b91180ff20d271fdac80ab											
456fb56e167a55e0a5783f279fad02ee											
6c40adf1ea6f603a98d4212378aa6ca6											

ASV	eDNA-209	eDNA-21	eDNA-210	eDNA-211	eDNA-212	eDNA-213	eDNA-214	eDNA-215	eDNA-216	eDNA-217	eDNA-218
0bd8aa353000d7200eab44f0f7f75d90											

ASV	eDNA-209	eDNA-21	eDNA-210	eDNA-211	eDNA-212	eDNA-213	eDNA-214	eDNA-215	eDNA-216	eDNA-217	eDNA-218
6a7be99afeee124f7a76f2998ebb597e											
7667427bcd6b201b6d6c106497acef37											
2bb407c368414e0969ccc76edf6c5c9d											
880459d1858aa106016e9cdd8c6e73e9		22			29						
59bcd49cb2e198f72833788401dcc7cb											
fc0f98f44f1bbe3177c059ae333e5c0f		20			7						
cc8940197219ad6ac1ce5759fe646fc1		2									
10d58e3b59f907c90e9dab491d2aae27											
bf51cc6e1dd2c7fde081c04a216c0172											
2c34cf16fe43896b9c58cc3bbc37f8e3											
66b1a8b2bc6d0b164e16ea88b1ec91bd											
9245b027bf154b1c4c9594ae843b26cf											
8d77fb3cc5c248e3376a9c0df5386bdf		26			15						
f83f71f93f6e908cb4f8cabd09a24cf8											
81a08c4056698cb10741d61e751704cc											
f691c9f1b907b4d8a601e493a5a358b2		3			3						
8f2fb4e5b8ffc59f7a1b7c1b995e25f4											
07f648cadd099e3c0494fc6550aabdca											
2ef7d952c54b153e64397fa47c24abff											
b87af5c73c103467cba30de2efed1929											
8f7dd9f608c87dd624f0d2429b923619											
fc5372f0ed9dde409b5b89f572c30840											
c63c60d9b4ab2d48cafd0786362c0a3c											
5c89d7b233dc5bc566aea8d8523050a8											
a961095895dc52b6e3810dbcc1fb9f91											
b331712b0c56a69a7fa95aa34017cfbd											
f625435363c3185d62cfad84d4b132e3											
794f0e4b71828ccdc76300b512ef2d7d		2									
4937cc30738dbb33464513afdebc8bcf											
b11e48466c55159b83352aaa222bc07e		4									
c09f21dfe23cf8b1f222672305d38944											

ASV	eDNA-209	eDNA-21	eDNA-210	eDNA-211	eDNA-212	eDNA-213	eDNA-214	eDNA-215	eDNA-216	eDNA-217	eDNA-218
b1c7e99bfa4a11334f2497d033d655bc											
78cc4d1a6e5a85ca641eda16a7fc78c9											
5d5142f1ec3aeed0c0da48a125934182											
c253a68c33b91180ff20d271fdac80ab											
456fb56e167a55e0a5783f279fad02ee											
6c40adf1ea6f603a98d4212378aa6ca6											

ASV	eDNA-219	eDNA-22	eDNA-220	eDNA-221	eDNA-222	eDNA-223	eDNA-224	eDNA-225	eDNA-226	eDNA-227	eDNA-228
0bd8aa353000d7200eab44f0f7f75d90											
6a7be99afeee124f7a76f2998ebb597e		1									
7667427bcd6b201b6d6c106497acef37											
2bb407c368414e0969ccc76edf6c5c9d											
880459d1858aa106016e9cdd8c6e73e9		29	6								30
59bcd49cb2e198f72833788401dcc7cb			1								
fc0f98f44f1bbe3177c059ae333e5c0f		19	5								24
cc8940197219ad6ac1ce5759fe646fc1											
10d58e3b59f907c90e9dab491d2aae27											
bf51cc6e1dd2c7fde081c04a216c0172											
2c34cf16fe43896b9c58cc3bbc37f8e3											
66b1a8b2bc6d0b164e16ea88b1ec91bd											
9245b027bf154b1c4c9594ae843b26cf											
8d77fb3cc5c248e3376a9c0df5386bdf		13	18								44
f83f71f93f6e908cb4f8cabd09a24cf8											1
81a08c4056698cb10741d61e751704cc											
f691c9f1b907b4d8a601e493a5a358b2		2									5
8f2fb4e5b8ffc59f7a1b7c1b995e25f4											
07f648cadd099e3c0494fc6550aabdca											
2ef7d952c54b153e64397fa47c24abff											
b87af5c73c103467cba30de2efed1929											
8f7dd9f608c87dd624f0d2429b923619											

ASV	eDNA-219	eDNA-22	eDNA-220	eDNA-221	eDNA-222	eDNA-223	eDNA-224	eDNA-225	eDNA-226	eDNA-227	eDNA-228
fc5372f0ed9dde409b5b89f572c30840											
c63c60d9b4ab2d48cafd0786362c0a3c											
5c89d7b233dc5bc566aea8d8523050a8											
a961095895dc52b6e3810dbcc1fb9f91											
b331712b0c56a69a7fa95aa34017cfbd											
f625435363c3185d62cfad84d4b132e3											
794f0e4b71828ccdc76300b512ef2d7d		2	2								4
4937cc30738dbb33464513afdebc8bcf											
b11e48466c55159b83352aaa222bc07e		2	1								2
c09f21dfe23cf8b1f222672305d38944											
b1c7e99bfa4a11334f2497d033d655bc											
78cc4d1a6e5a85ca641eda16a7fc78c9											
5d5142f1ec3aeed0c0da48a125934182											
c253a68c33b91180ff20d271fdac80ab											
456fb56e167a55e0a5783f279fad02ee											
6c40adf1ea6f603a98d4212378aa6ca6											

ASV	eDNA-229	eDNA-23	eDNA-230	eDNA-231	eDNA-232	eDNA-233	eDNA-234	eDNA-235	eDNA-236	eDNA-237	eDNA-238
0bd8aa353000d7200eab44f0f7f75d90											
6a7be99afeee124f7a76f2998ebb597e											
7667427bcd6b201b6d6c106497acef37											
2bb407c368414e0969ccc76edf6c5c9d											
880459d1858aa106016e9cdd8c6e73e9		16							25		
59bcd49cb2e198f72833788401dcc7cb											
fc0f98f44f1bbe3177c059ae333e5c0f		12							17		
cc8940197219ad6ac1ce5759fe646fc1											
10d58e3b59f907c90e9dab491d2aae27											
bf51cc6e1dd2c7fde081c04a216c0172											
2c34cf16fe43896b9c58cc3bbc37f8e3											
66b1a8b2bc6d0b164e16ea88b1ec91bd											

ASV	eDNA-229	eDNA-23	eDNA-230	eDNA-231	eDNA-232	eDNA-233	eDNA-234	eDNA-235	eDNA-236	eDNA-237	eDNA-238
9245b027bf154b1c4c9594ae843b26cf											
8d77fb3cc5c248e3376a9c0df5386bdf		10							15		
f83f71f93f6e908cb4f8cabd09a24cf8									1		
81a08c4056698cb10741d61e751704cc											
f691c9f1b907b4d8a601e493a5a358b2		3							2		
8f2fb4e5b8ffc59f7a1b7c1b995e25f4									1		
07f648cadd099e3c0494fc6550aabdca											
2ef7d952c54b153e64397fa47c24abff											
b87af5c73c103467cba30de2efed1929											
8f7dd9f608c87dd624f0d2429b923619											
fc5372f0ed9dde409b5b89f572c30840											
c63c60d9b4ab2d48cafd0786362c0a3c											
5c89d7b233dc5bc566aea8d8523050a8											
a961095895dc52b6e3810dbcc1fb9f91									1		
b331712b0c56a69a7fa95aa34017cfbd											
f625435363c3185d62cfad84d4b132e3											
794f0e4b71828ccdc76300b512ef2d7d									4		
4937cc30738dbb33464513afdebc8bcf											
b11e48466c55159b83352aaa222bc07e									1		
c09f21dfe23cf8b1f222672305d38944											
b1c7e99bfa4a11334f2497d033d655bc											
78cc4d1a6e5a85ca641eda16a7fc78c9											
5d5142f1ec3aeed0c0da48a125934182											
c253a68c33b91180ff20d271fdac80ab											
456fb56e167a55e0a5783f279fad02ee											
6c40adf1ea6f603a98d4212378aa6ca6											

ASV	eDNA-239	eDNA-24	eDNA-240	eDNA-241	eDNA-242	eDNA-243	eDNA-244	eDNA-245	eDNA-246	eDNA-247	eDNA-248
0bd8aa353000d7200eab44f0f7f75d90											
6a7be99afeee124f7a76f2998ebb597e											

ASV	eDNA-239	eDNA-24	eDNA-240	eDNA-241	eDNA-242	eDNA-243	eDNA-244	eDNA-245	eDNA-246	eDNA-247	eDNA-248
7667427bcd6b201b6d6c106497acef37											
2bb407c368414e0969ccc76edf6c5c9d											
880459d1858aa106016e9cdd8c6e73e9		10					9	7	6	1	6
59bcd49cb2e198f72833788401dcc7cb											
fc0f98f44f1bbe3177c059ae333e5c0f		10					19		12		2
cc8940197219ad6ac1ce5759fe646fc1											
10d58e3b59f907c90e9dab491d2aae27											
bf51cc6e1dd2c7fde081c04a216c0172		2					1				
2c34cf16fe43896b9c58cc3bbc37f8e3											
66b1a8b2bc6d0b164e16ea88b1ec91bd										2	
9245b027bf154b1c4c9594ae843b26cf											
8d77fb3cc5c248e3376a9c0df5386bdf		9					15		7		12
f83f71f93f6e908cb4f8cabd09a24cf8											
81a08c4056698cb10741d61e751704cc											
f691c9f1b907b4d8a601e493a5a358b2		2					5				1
8f2fb4e5b8ffc59f7a1b7c1b995e25f4		1									
07f648cadd099e3c0494fc6550aabdca											
2ef7d952c54b153e64397fa47c24abff											
b87af5c73c103467cba30de2efed1929											
8f7dd9f608c87dd624f0d2429b923619											
fc5372f0ed9dde409b5b89f572c30840											
c63c60d9b4ab2d48cafd0786362c0a3c											
5c89d7b233dc5bc566aea8d8523050a8											
a961095895dc52b6e3810dbcc1fb9f91											
b331712b0c56a69a7fa95aa34017cfbd											
f625435363c3185d62cfad84d4b132e3											
794f0e4b71828ccdc76300b512ef2d7d		3									
4937cc30738dbb33464513afdebc8bcf											
b11e48466c55159b83352aaa222bc07e		2					1			1	
c09f21dfe23cf8b1f222672305d38944											
b1c7e99bfa4a11334f2497d033d655bc											

ASV	eDNA-239	eDNA-24	eDNA-240	eDNA-241	eDNA-242	eDNA-243	eDNA-244	eDNA-245	eDNA-246	eDNA-247	eDNA-248
78cc4d1a6e5a85ca641eda16a7fc78c9											
5d5142f1ec3aeed0c0da48a125934182											
c253a68c33b91180ff20d271fdac80ab											
456fb56e167a55e0a5783f279fad02ee											
6c40adf1ea6f603a98d4212378aa6ca6											

ASV	eDNA-249	eDNA-25	eDNA-250	eDNA-251	eDNA-26	eDNA-27	eDNA-28	eDNA-29	eDNA-3	eDNA-30	eDNA-31
0bd8aa353000d7200eab44f0f7f75d90											
6a7be99afeee124f7a76f2998ebb597e											
7667427bcd6b201b6d6c106497acef37											
2bb407c368414e0969ccc76edf6c5c9d											
880459d1858aa106016e9cdd8c6e73e9	1	12						38		28	24
59bcd49cb2e198f72833788401dcc7cb											
fc0f98f44f1bbe3177c059ae333e5c0f		14		2				25		14	9
cc8940197219ad6ac1ce5759fe646fc1											1
10d58e3b59f907c90e9dab491d2aae27											
bf51cc6e1dd2c7fde081c04a216c0172		3									2
2c34cf16fe43896b9c58cc3bbc37f8e3											
66b1a8b2bc6d0b164e16ea88b1ec91bd											
9245b027bf154b1c4c9594ae843b26cf											
8d77fb3cc5c248e3376a9c0df5386bdf		10		4				43		27	15
f83f71f93f6e908cb4f8cabd09a24cf8								1		2	1
81a08c4056698cb10741d61e751704cc											
f691c9f1b907b4d8a601e493a5a358b2		4	2					4		5	2
8f2fb4e5b8ffc59f7a1b7c1b995e25f4											
07f648cadd099e3c0494fc6550aabdca											
2ef7d952c54b153e64397fa47c24abff											
b87af5c73c103467cba30de2efed1929											
8f7dd9f608c87dd624f0d2429b923619											
fc5372f0ed9dde409b5b89f572c30840											

ASV	eDNA-249	eDNA-25	eDNA-250	eDNA-251	eDNA-26	eDNA-27	eDNA-28	eDNA-29	eDNA-3	eDNA-30	eDNA-31
c63c60d9b4ab2d48cafd0786362c0a3c											
5c89d7b233dc5bc566aea8d8523050a8											
a961095895dc52b6e3810dbcc1fb9f91								1			
b331712b0c56a69a7fa95aa34017cfbd											
f625435363c3185d62cfad84d4b132e3											
794f0e4b71828ccdc76300b512ef2d7d		2						2		2	1
4937cc30738dbb33464513afdebc8bcf											
b11e48466c55159b83352aaa222bc07e	1	2						3			3
c09f21dfe23cf8b1f222672305d38944											
b1c7e99bfa4a11334f2497d033d655bc											
78cc4d1a6e5a85ca641eda16a7fc78c9											
5d5142f1ec3aeed0c0da48a125934182											
c253a68c33b91180ff20d271fdac80ab											
456fb56e167a55e0a5783f279fad02ee											
6c40adf1ea6f603a98d4212378aa6ca6											

ASV	eDNA-32	eDNA-33	eDNA-34	eDNA-35	eDNA-36	eDNA-37	eDNA-38	eDNA-39	eDNA-4	eDNA-40	eDNA-41
0bd8aa353000d7200eab44f0f7f75d90											
6a7be99afeee124f7a76f2998ebb597e											
7667427bcd6b201b6d6c106497acef37											
2bb407c368414e0969ccc76edf6c5c9d											
880459d1858aa106016e9cdd8c6e73e9	19	10				39	47	20		26	9
59bcd49cb2e198f72833788401dcc7cb											
fc0f98f44f1bbe3177c059ae333e5c0f	13	10				25	18	9		8	5
cc8940197219ad6ac1ce5759fe646fc1	1							1			
10d58e3b59f907c90e9dab491d2aae27											
bf51cc6e1dd2c7fde081c04a216c0172	2					2				2	1
2c34cf16fe43896b9c58cc3bbc37f8e3											
66b1a8b2bc6d0b164e16ea88b1ec91bd											
9245b027bf154b1c4c9594ae843b26cf											

ASV	eDNA-32	eDNA-33	eDNA-34	eDNA-35	eDNA-36	eDNA-37	eDNA-38	eDNA-39	eDNA-4	eDNA-40	eDNA-41
8d77fb3cc5c248e3376a9c0df5386bdf	21	7				46	34	18		10	12
f83f71f93f6e908cb4f8cabd09a24cf8	1	2				1	1			1	
81a08c4056698cb10741d61e751704cc											
f691c9f1b907b4d8a601e493a5a358b2		2				5	4	4		6	1
8f2fb4e5b8ffc59f7a1b7c1b995e25f4											
07f648cadd099e3c0494fc6550aabdca											
2ef7d952c54b153e64397fa47c24abff											
b87af5c73c103467cba30de2efed1929											
8f7dd9f608c87dd624f0d2429b923619											
fc5372f0ed9dde409b5b89f572c30840											
c63c60d9b4ab2d48cafd0786362c0a3c											
5c89d7b233dc5bc566aea8d8523050a8											
a961095895dc52b6e3810dbcc1fb9f91											
b331712b0c56a69a7fa95aa34017cfbd											
f625435363c3185d62cfad84d4b132e3											
794f0e4b71828ccdc76300b512ef2d7d						5		2			
4937cc30738dbb33464513afdebc8bcf				1	2						
b11e48466c55159b83352aaa222bc07e	5	1				4		1		1	1
c09f21dfe23cf8b1f222672305d38944											
b1c7e99bfa4a11334f2497d033d655bc											
78cc4d1a6e5a85ca641eda16a7fc78c9											
5d5142f1ec3aeed0c0da48a125934182											
c253a68c33b91180ff20d271fdac80ab											
456fb56e167a55e0a5783f279fad02ee											
6c40adf1ea6f603a98d4212378aa6ca6											

ASV	eDNA-42-MS	eDNA-42	eDNA-43	eDNA-44	eDNA-46	eDNA-47	eDNA-48	eDNA-49	eDNA-5	eDNA-50	eDNA-51
0bd8aa353000d7200eab44f0f7f75d90	18										
6a7be99afeee124f7a76f2998ebb597e	23										
7667427bcd6b201b6d6c106497acef37											

ASV	eDNA-42-MS	eDNA-42	eDNA-43	eDNA-44	eDNA-46	eDNA-47	eDNA-48	eDNA-49	eDNA-5	eDNA-50	eDNA-51
2bb407c368414e0969ccc76edf6c5c9d	9										
880459d1858aa106016e9cdd8c6e73e9	38638				12	9	10	36	15		
59bcd49cb2e198f72833788401dcc7cb											
fc0f98f44f1bbe3177c059ae333e5c0f	24956				12	5	2	18	22		
cc8940197219ad6ac1ce5759fe646fc1	761					2					
10d58e3b59f907c90e9dab491d2aae27											
bf51cc6e1dd2c7fde081c04a216c0172	1030			1	1		1				
2c34cf16fe43896b9c58cc3bbc37f8e3	41										
66b1a8b2bc6d0b164e16ea88b1ec91bd											
9245b027bf154b1c4c9594ae843b26cf	2										
8d77fb3cc5c248e3376a9c0df5386bdf	38910				20	7	10	27	22		
f83f71f93f6e908cb4f8cabd09a24cf8	2023				1	1			1		
81a08c4056698cb10741d61e751704cc	5										
f691c9f1b907b4d8a601e493a5a358b2	6220				3	2	1	5	2		
8f2fb4e5b8ffc59f7a1b7c1b995e25f4	326										
07f648cadd099e3c0494fc6550aabdca	2										
2ef7d952c54b153e64397fa47c24abff	366					3					
b87af5c73c103467cba30de2efed1929											
8f7dd9f608c87dd624f0d2429b923619											
fc5372f0ed9dde409b5b89f572c30840	8										
c63c60d9b4ab2d48cafd0786362c0a3c	3										
5c89d7b233dc5bc566aea8d8523050a8											
a961095895dc52b6e3810dbcc1fb9f91	44										
b331712b0c56a69a7fa95aa34017cfbd											
f625435363c3185d62cfad84d4b132e3											
794f0e4b71828ccdc76300b512ef2d7d	3995				2	1	2	3	4		
4937cc30738dbb33464513afdebc8bcf				1							
b11e48466c55159b83352aaa222bc07e	2445					2		1	2		
c09f21dfe23cf8b1f222672305d38944	32										
b1c7e99bfa4a11334f2497d033d655bc											
78cc4d1a6e5a85ca641eda16a7fc78c9	14										

ASV	eDNA-42-MS	eDNA-42	eDNA-43	eDNA-44	eDNA-46	eDNA-47	eDNA-48	eDNA-49	eDNA-5	eDNA-50	eDNA-51
5d5142f1ec3aeed0c0da48a125934182	62										
c253a68c33b91180ff20d271fdac80ab											
456fb56e167a55e0a5783f279fad02ee											
6c40adf1ea6f603a98d4212378aa6ca6	17										

ASV	eDNA-52	eDNA-53	eDNA-54	eDNA-55	eDNA-56-MS	eDNA-56	eDNA-57	eDNA-58	eDNA-59	eDNA-6	eDNA-60
0bd8aa353000d7200eab44f0f7f75d90					49						
6a7be99afeee124f7a76f2998ebb597e					27						
7667427bcd6b201b6d6c106497acef37					2						
2bb407c368414e0969ccc76edf6c5c9d					25						
880459d1858aa106016e9cdd8c6e73e9		69	66	31	57185	28	5			9	
59bcd49cb2e198f72833788401dcc7cb											
fc0f98f44f1bbe3177c059ae333e5c0f		49	47	25	34424	29	4			10	
cc8940197219ad6ac1ce5759fe646fc1					1261	1					
10d58e3b59f907c90e9dab491d2aae27					4						
bf51cc6e1dd2c7fde081c04a216c0172		1	2	1	1639						
2c34cf16fe43896b9c58cc3bbc37f8e3					36						
66b1a8b2bc6d0b164e16ea88b1ec91bd											
9245b027bf154b1c4c9594ae843b26cf					1						
8d77fb3cc5c248e3376a9c0df5386bdf		52	71	30	55299	25	10			13	
f83f71f93f6e908cb4f8cabd09a24cf8		6			2646		2				
81a08c4056698cb10741d61e751704cc					7						
f691c9f1b907b4d8a601e493a5a358b2		13	8	3	9815	7				3	
8f2fb4e5b8ffc59f7a1b7c1b995e25f4		2			450						
07f648cadd099e3c0494fc6550aabdca											
2ef7d952c54b153e64397fa47c24abff					582						
b87af5c73c103467cba30de2efed1929										42	
8f7dd9f608c87dd624f0d2429b923619											
fc5372f0ed9dde409b5b89f572c30840					52						
c63c60d9b4ab2d48cafd0786362c0a3c					1						

ASV	eDNA-52	eDNA-53	eDNA-54	eDNA-55	eDNA-56-MS	eDNA-56	eDNA-57	eDNA-58	eDNA-59	eDNA-6	eDNA-60
5c89d7b233dc5bc566aea8d8523050a8											
a961095895dc52b6e3810dbcc1fb9f91					113						
b331712b0c56a69a7fa95aa34017cfbd					72						
f625435363c3185d62cfad84d4b132e3											
794f0e4b71828ccdc76300b512ef2d7d		5	4	6	5906	3				3	
4937cc30738dbb33464513afdebc8bcf	5										1
b11e48466c55159b83352aaa222bc07e		5	3	4	4224	1					
c09f21dfe23cf8b1f222672305d38944					27						
b1c7e99bfa4a11334f2497d033d655bc											
78cc4d1a6e5a85ca641eda16a7fc78c9					13						
5d5142f1ec3aeed0c0da48a125934182		2			121						
c253a68c33b91180ff20d271fdac80ab					36						
456fb56e167a55e0a5783f279fad02ee											
6c40adf1ea6f603a98d4212378aa6ca6					39						

ASV	eDNA-61	eDNA-62	eDNA-63	eDNA-64	eDNA-65	eDNA-66	eDNA-67	eDNA-68	eDNA-69-MS	eDNA-69	eDNA-7
0bd8aa353000d7200eab44f0f7f75d90									15		
6a7be99afeee124f7a76f2998ebb597e									11		
7667427bcd6b201b6d6c106497acef37											
2bb407c368414e0969ccc76edf6c5c9d									5		
880459d1858aa106016e9cdd8c6e73e9	26	16	13	11	9				45439	33	9
59bcd49cb2e198f72833788401dcc7cb											
fc0f98f44f1bbe3177c059ae333e5c0f	17	13	10	4	4				29458	18	4
cc8940197219ad6ac1ce5759fe646fc1	1			1					855	1	
10d58e3b59f907c90e9dab491d2aae27											
bf51cc6e1dd2c7fde081c04a216c0172		2			1				1369		
2c34cf16fe43896b9c58cc3bbc37f8e3									33		
66b1a8b2bc6d0b164e16ea88b1ec91bd											
9245b027bf154b1c4c9594ae843b26cf									2		
8d77fb3cc5c248e3376a9c0df5386bdf	20	12	13	11	6				43160	19	8

ASV	eDNA-61	eDNA-62	eDNA-63	eDNA-64	eDNA-65	eDNA-66	eDNA-67	eDNA-68	eDNA-69-MS	eDNA-69	eDNA-7
f83f71f93f6e908cb4f8cabd09a24cf8									2218	1	
81a08c4056698cb10741d61e751704cc									2		
f691c9f1b907b4d8a601e493a5a358b2	5								8529	2	
8f2fb4e5b8ffc59f7a1b7c1b995e25f4									312	3	
07f648cadd099e3c0494fc6550aabdca											
2ef7d952c54b153e64397fa47c24abff									384	1	
b87af5c73c103467cba30de2efed1929											
8f7dd9f608c87dd624f0d2429b923619											
fc5372f0ed9dde409b5b89f572c30840									5		
c63c60d9b4ab2d48cafd0786362c0a3c									4		
5c89d7b233dc5bc566aea8d8523050a8											
a961095895dc52b6e3810dbcc1fb9f91									46		
b331712b0c56a69a7fa95aa34017cfbd											
f625435363c3185d62cfad84d4b132e3											
794f0e4b71828ccdc76300b512ef2d7d	2	2			1				4774	4	
4937cc30738dbb33464513afdebc8bcf							30				
b11e48466c55159b83352aaa222bc07e				2	4				3081		
c09f21dfe23cf8b1f222672305d38944											
b1c7e99bfa4a11334f2497d033d655bc									22		
78cc4d1a6e5a85ca641eda16a7fc78c9									7		
5d5142f1ec3aeed0c0da48a125934182									28		
c253a68c33b91180ff20d271fdac80ab											
456fb56e167a55e0a5783f279fad02ee											
6c40adf1ea6f603a98d4212378aa6ca6									2		

ASV	eDNA-70	eDNA-71	eDNA-72	eDNA-73	eDNA-74	eDNA-75	eDNA-76	eDNA-77	eDNA-78	eDNA-79	eDNA-8
0bd8aa353000d7200eab44f0f7f75d90											
6a7be99afeee124f7a76f2998ebb597e							3				
7667427bcd6b201b6d6c106497acef37											
2bb407c368414e0969ccc76edf6c5c9d											

ASV	eDNA-70	eDNA-71	eDNA-72	eDNA-73	eDNA-74	eDNA-75	eDNA-76	eDNA-77	eDNA-78	eDNA-79	eDNA-8
0bd8aa353000d7200eab44f0f7f75d90											
880459d1858aa106016e9cdd8c6e73e9	26	6	12	14			59	48	26	11	3
59bcd49cb2e198f72833788401dcc7cb											
fc0f98f44f1bbe3177c059ae333e5c0f	14	12	120	100	20		333	192	23	10	4
cc8940197219ad6ac1ce5759fe646fc1											1
10d58e3b59f907c90e9dab491d2aae27											
bf51cc6e1dd2c7fde081c04a216c0172		1		13	3		109				
2c34cf16fe43896b9c58cc3bbc37f8e3											
66b1a8b2bc6d0b164e16ea88b1ec91bd											
9245b027bf154b1c4c9594ae843b26cf											
8d77fb3cc5c248e3376a9c0df5386bdf	15	10	9	9				35	20	10	10
f83f71f93f6e908cb4f8cabd09a24cf8		3						3		1	
81a08c4056698cb10741d61e751704cc											
f691c9f1b907b4d8a601e493a5a358b2	2	2	2	1				6	6	3	3
8f2fb4e5b8ffc59f7a1b7c1b995e25f4											
07f648cadd099e3c0494fc6550aabdca											
2ef7d952c54b153e64397fa47c24abff											
b87af5c73c103467cba30de2efed1929											
8f7dd9f608c87dd624f0d2429b923619											
fc5372f0ed9dde409b5b89f572c30840											
c63c60d9b4ab2d48cafd0786362c0a3c											
5c89d7b233dc5bc566aea8d8523050a8											
a961095895dc52b6e3810dbcc1fb9f91											
b331712b0c56a69a7fa95aa34017cfbd											
f625435363c3185d62cfad84d4b132e3											
794f0e4b71828ccdc76300b512ef2d7d	4	1						4	1		
4937cc30738dbb33464513afdebc8bcf			53	225	97	545	1710	64			
b11e48466c55159b83352aaa222bc07e		2	1						4		
c09f21dfe23cf8b1f222672305d38944											
b1c7e99bfa4a11334f2497d033d655bc											
78cc4d1a6e5a85ca641eda16a7fc78c9											

ASV	eDNA-70	eDNA-71	eDNA-72	eDNA-73	eDNA-74	eDNA-75	eDNA-76	eDNA-77	eDNA-78	eDNA-79	eDNA-8
0bd8aa353000d7200eab44f0f7f75d90											
5d5142f1ec3aeed0c0da48a125934182											
c253a68c33b91180ff20d271fdac80ab											
456fb56e167a55e0a5783f279fad02ee							60				
6c40adf1ea6f603a98d4212378aa6ca6											

ASV	eDNA-80	eDNA-81	eDNA-82	eDNA-83	eDNA-84	eDNA-9	eDNA-M1	eDNA-M2	Total_reads	TaxonomyID
0bd8aa353000d7200eab44f0f7f75d90							49	58	189	Tachyglossus
6a7be99afeee124f7a76f2998ebb597e							37	33	135	Canis
7667427bcd6b201b6d6c106497acef37									2	Lagorchestes
2bb407c368414e0969ccc76edf6c5c9d							27	23	89	Petrogale
880459d1858aa106016e9cdd8c6e73e9	13	49	9	9	18	11	71453	69343	283423	Tachyglossus
59bcd49cb2e198f72833788401dcc7cb									1	Tachyglossus
fc0f98f44f1bbe3177c059ae333e5c0f	15	22	6	2	6	12	42245	42347	175036	Canis
cc8940197219ad6ac1ce5759fe646fc1						1	1137	1226	5257	Lagorchestes
10d58e3b59f907c90e9dab491d2aae27									4	Zyzomys
bf51cc6e1dd2c7fde081c04a216c0172		2					1686	1772	7732	Zyzomys
2c34cf16fe43896b9c58cc3bbc37f8e3							61	55	226	Wyulda
66b1a8b2bc6d0b164e16ea88b1ec91bd									2	Wyulda
9245b027bf154b1c4c9594ae843b26cf							6	2	13	Pseudantechinus
8d77fb3cc5c248e3376a9c0df5386bdf	15	47	7	4	8	6	72706	71135	282392	Wyulda
f83f71f93f6e908cb4f8cabd09a24cf8	1	2		1			3745	3625	14295	Pseudantechinus
81a08c4056698cb10741d61e751704cc							8	6	28	Pseudomys
f691c9f1b907b4d8a601e493a5a358b2		7				2	10670	11432	46851	Pseudomys
8f2fb4e5b8ffc59f7a1b7c1b995e25f4							556	525	2176	Rattus
07f648cadd099e3c0494fc6550aabdca									2	Planigale
2ef7d952c54b153e64397fa47c24abff							722	645	2705	Planigale
b87af5c73c103467cba30de2efed1929									42	Canis
8f7dd9f608c87dd624f0d2429b923619								65	65	Trichosurus
fc5372f0ed9dde409b5b89f572c30840							46	35	146	Canis

ASV	eDNA-80	eDNA-81	eDNA-82	eDNA-83	eDNA-84	eDNA-9	eDNA-M1	eDNA-M2	Total_reads	TaxonomyID
c63c60d9b4ab2d48cafd0786362c0a3c							8	10	26	Trichosurus
5c89d7b233dc5bc566aea8d8523050a8							2		2	Zyzomys
a961095895dc52b6e3810dbcc1fb9f91							109	116	430	Muridae
b331712b0c56a69a7fa95aa34017cfbd								52	124	Trichosurus
f625435363c3185d62cfad84d4b132e3							76	61	137	Phalangeridae
794f0e4b71828ccdc76300b512ef2d7d	1	4		1		2	7062	7010	28845	Trichosurus
4937cc30738dbb33464513afdebc8bcf		1		2	9				2749	Diprotodontia
b11e48466c55159b83352aaa222bc07e		2		2			4152	4415	18394	Zyzomys
c09f21dfe23cf8b1f222672305d38944							43	42	144	Mammalia
b1c7e99bfa4a11334f2497d033d655bc									22	Trichosurus
78cc4d1a6e5a85ca641eda16a7fc78c9							36	33	103	Pseudantechinus
5d5142f1ec3aeed0c0da48a125934182							101	87	401	Zyzomys
c253a68c33b91180ff20d271fdac80ab									36	Mammalia
456fb56e167a55e0a5783f279fad02ee									60	Diprotodontia
6c40adf1ea6f603a98d4212378aa6ca6							33	27	118	Dasyuridae

ASV	Confidence(%)	Sequence
0bd8aa353000d7200eab44f0f7f75d90	100	AAACTAATTGAGTAAAAATTTTAGTTATTATACCCTTAAGTTGAATGTTTGAAGAAGTTACTCTTTAATTAA
6a7be99afeee124f7a76f2998ebb597e	100	AAATTGCTAACTCATAATAATGGTGTTATGTTATGCCTTGTAGGTATCTAGTATCCATAAGTTTGGGTTAGTTAATTAA
7667427bcd6b201b6d6c106497acef37	100	AAGGTTATAGTCTAGTAGGTCTGGATTTGTTCCTGTGAGTAGGGTAATGTTGTTTGAATTAGACTATGAATCTTA
2bb407c368414e0969ccc76edf6c5c9d	100	TAAGATTCATAGTCTAATTCGAAATATTTACACCCCTAAATACAGGAACAAAATCAGACCTACTAGACTATAACCTT
880459d1858aa106016e9cdd8c6e73e9	100	TTAATTAAAGAGTAACTTCTTCAAACATTCAACTTAAGGGTATAATAACTAAAATTTTTACTCAATTAGTTT
59bcd49cb2e198f72833788401dcc7cb	100	TTAATTAAAGAGTAACTTCTTCAAACATTCAACTTAAGGGTATAATAACTAAAATTTTTACTCAATTAGTTTC
fc0f98f44f1bbe3177c059ae333e5c0f	100	TTAATTAACTAACCCAAACTTATGGATACTAGATACCTACAAGGCATAACATAACACCATTATTATGAGTTAGCAATTT
cc8940197219ad6ac1ce5759fe646fc1	98	TAAGATTCATAGTCTAATTCAAACAACATTACCCTACTCACAGGAACAAATCCAGACCTACTAGACTATAACCTT
10d58e3b59f907c90e9dab491d2aae27	96	AAATTTTGAGCTTAATATTTATTGTGTTATGCCATTAGGTTATTTAAATATATTAATTAAGCTTTTAATTTA
bf51cc6e1dd2c7fde081c04a216c0172	95	TAAATTAAAAGCTTAATTAATATATTTAAATAACCTAATGGCATAACACAATAAATATTAAGCTCAAAATTT
2c34cf16fe43896b9c58cc3bbc37f8e3	94	AAGGTTATAGTCTAGTTGGTTATAGTGTTGTTCCATGTAGGGTTAAATTTGGTTAGATTAGACTATGAATCTTA
66b1a8b2bc6d0b164e16ea88b1ec91bd	91	TAAGATTCATAGTCTAATCTAACCAAATTTAACCCTACATGGAACAACACTATAACCAACTAGACTATAACCTTT
9245b027bf154b1c4c9594ae843b26cf	88	AAGACTATAACTCAGGTGTTTATACATATTGTTCCTGTAGGTTGAATGTTCGTATGTGTTGAGTTATGTGACTTA

ASV	Confidence(%)	Sequence
8d77fb3cc5c248e3376a9c0df5386bdf	88	TAAGATTCATAGTCTAATCTAACCAAATTTAACCCTACATGGAACAACACTATAACCAACTAGACTATAACCTT
f83f71f93f6e908cb4f8cabd09a24cf8	86	TAAGTCACATAACTCAACACATACGAACATTCAACCTACAGGAACAATATGTATAAACACCTGAGTTATAGTCTT
81a08c4056698cb10741d61e751704cc	83	AAATTATAAGTTTAGTATTTTTGTGTTGAGCCAATTAGGTTTTGTAGATGCGAAAATTAAACTTTTAATTTA
f691c9f1b907b4d8a601e493a5a358b2	83	TAAATTAAAAGTTTAATTTTCGCATCTACAAAACCTAATTGGCTCAACACAAAAATACTAAACTTATAATTT
8f2fb4e5b8ffc59f7a1b7c1b995e25f4	83	TTAATTTACTAGTTTAACTTATGCATAATAACCTAATGGACCAAAACAACATAATCATAAACTAAAAATTT
07f648cadd099e3c0494fc6550aabdca	79	AAGACTATAGCTCAGGACTTTATACATTTTTGTCCCGGTAGGTTTAATTAGGTATGTGTTGAGCTATGTGTCTTA
2ef7d952c54b153e64397fa47c24abff	77	TAAGACACATAGCTCAACACATACCTAATTAAACCTACCGGGACAAAAATGTATAAAGTCCTGAGCTATAGTCTT
b87af5c73c103467cba30de2efed1929	75	TTAATTAACTAACCCAAACTCATGGATACTAGATACCTACAAGGCATAACATAACACCATTATTATGGGTTAGCAATTT
8f7dd9f608c87dd624f0d2429b923619	74	TAAGATTCATAGTCTAACCTAACTCAACAAAACCCTACATTGAACAATACAATGACTAACTAGACTATAACCTT
fc5372f0ed9dde409b5b89f572c30840	74	TTAATTAACTAACCCAAACTTATGGATACTAAATACCTATAAGGCATAACATAACACCATTATTATGGGTTAGCAATTT
c63c60d9b4ab2d48cafd0786362c0a3c	71	AAGGTTATAGTCTAGTTAGTCATTGTATTGTTCCATGTAGGGTTTTGTTGAGTTAGGTTAGACTATGAATCTTA
5c89d7b233dc5bc566aea8d8523050a8	68	AAATTTTGAGTTTAATATTTATTGTGTTATTGCCATTAGGTTATTTAAATATATTAATTAAACTTTTAATTTA
a961095895dc52b6e3810dbcc1fb9f91	66	TAAATTAAAAGTTTAATTAATATATTCAAATAGCCTAATGGCATAACACAATAAATATTAAACTCAAAATTT
b331712b0c56a69a7fa95aa34017cfbd	65	TAAGATTCATAGTCTAACCTAACTAAACAAAACCCTACATGGAACAATACAATGACTAACTAGACTATAACCTT
f625435363c3185d62cfad84d4b132e3	64	TAAGATTCATAGTCTAACCTAACTCAACAAAACCATACATGGAACAATACAATGACTAACTAGACTATAACCTT
794f0e4b71828ccdc76300b512ef2d7d	63	TAAGATTCATAGTCTAACCTAACTCAACAAAACCCTACATGGAACAATACAATGACTAACTAGACTATAACCTT
4937cc30738dbb33464513afdebc8bcf	63	TAAGATTCATAGTCTAATTCAAACAATTCTACCCTACATACAGGAACAAACACAGACCTACTAGACTATAACCTT
b11e48466c55159b83352aaa222bc07e	62	TAAATTAAAAGTTTAATTAATATATTTAAATAACCTAATGGCAATAACACAATAAATATTAAACTCAAAATTT
c09f21dfe23cf8b1f222672305d38944	62	TAAGATTCATAGTCTAACCTAACCCAACAAAACCCTACATGGAACAATATAATGACTAACTAGACTATAACCTT
b1c7e99bfa4a11334f2497d033d655bc	61	TAATATTCATAGTCTAACCTAACTCAACAAAACCCTACATTGAACAATACAATGACTAACTAGACTATAACCTT
78cc4d1a6e5a85ca641eda16a7fc78c9	60	TAAATCGCATAACTCAACACATACGAACATTCAACCTACAGGAACAATACGTATAAACACCTGAGTTATAGTCTT
5d5142f1ec3aeed0c0da48a125934182	60	TAAATTAAAAGTTTAATTAATATATTTAAACAGCCTAATGGCATAACACAATAAATATTAAACTTAAAATTT
c253a68c33b91180ff20d271fdac80ab	60	TAAGACACATAGCTCAACACATACTTAATTAAACCTACCGGGACAAAAATGTATAAAGTTCTGAGCTATAGTCTT
456fb56e167a55e0a5783f279fad02ee	60	TAAGATTCATAGTCTAATTCAAACAATTCTACCCTACATACAGGAACAAAAACAGACCTACTAGACTATAACCTT
6c40adf1ea6f603a98d4212378aa6ca6	60	TAAGTCGCATAACTCAACACATACGAACATTCAACCTACAGGAACAATACGTATAAACACCTGAGTTATAGTCTT

Table 3: Aves02_counts.

ASV	eDNA-1	eDNA-10	eDNA-11	eDNA-12	eDNA-13	eDNA-14	eDNA-15	eDNA-157	eDNA-158	eDNA-159
1042ffb53db165942e8f58b1a5c1cc1e										
7adfb35044222beddd25d5535887890f					15	18	7			
f78b7cad11793316ad17f6da1ecb9ec6										
661b0653a6a675f8e7d63c97e61f08fd										
5b8d404061fddbafd74b60fa35b99cc2					1					
82b75e5ee10a2d01e9cd73c5113cbe41										
0a0d571db32d0a34e760552eca16af9d										

ASV	eDNA-16	eDNA-160	eDNA-161	eDNA-162	eDNA-163	eDNA-164	eDNA-165	eDNA-166	eDNA-167	eDNA-168	eDNA-169
1042ffb53db165942e8f58b1a5c1cc1e											
7adfb35044222beddd25d5535887890f	3					15					
f78b7cad11793316ad17f6da1ecb9ec6											
661b0653a6a675f8e7d63c97e61f08fd											
5b8d404061fddbafd74b60fa35b99cc2						2					
82b75e5ee10a2d01e9cd73c5113cbe41											
0a0d571db32d0a34e760552eca16af9d											

ASV	eDNA-17	eDNA-170	eDNA-171	eDNA-172	eDNA-173	eDNA-174	eDNA-175	eDNA-176	eDNA-177	eDNA-178	eDNA-179
1042ffb53db165942e8f58b1a5c1cc1e											
7adfb35044222beddd25d5535887890f	2										
f78b7cad11793316ad17f6da1ecb9ec6											
661b0653a6a675f8e7d63c97e61f08fd											
5b8d404061fddbafd74b60fa35b99cc2											
82b75e5ee10a2d01e9cd73c5113cbe41											
0a0d571db32d0a34e760552eca16af9d											

ASV	eDNA-18	eDNA-180	eDNA-181	eDNA-182	eDNA-183	eDNA-184	eDNA-185	eDNA-186	eDNA-187	eDNA-188	eDNA-189
1042ffb53db165942e8f58b1a5c1cc1e											
7adfb35044222beddd25d5535887890f		13								19	
f78b7cad11793316ad17f6da1ecb9ec6											
661b0653a6a675f8e7d63c97e61f08fd											
5b8d404061fddbafd74b60fa35b99cc2		2								1	
82b75e5ee10a2d01e9cd73c5113cbe41											
0a0d571db32d0a34e760552eca16af9d											

ASV	eDNA-19	eDNA-190	eDNA-191	eDNA-192	eDNA-193	eDNA-194	eDNA-195	eDNA-196	eDNA-197	eDNA-198	eDNA-199
1042ffb53db165942e8f58b1a5c1cc1e											
7adfb35044222beddd25d5535887890f								12			
f78b7cad11793316ad17f6da1ecb9ec6											2
661b0653a6a675f8e7d63c97e61f08fd											
5b8d404061fddbafd74b60fa35b99cc2								2			
82b75e5ee10a2d01e9cd73c5113cbe41											
0a0d571db32d0a34e760552eca16af9d											

ASV	eDNA-2	eDNA-20	eDNA-200	eDNA-201	eDNA-202	eDNA-203	eDNA-204	eDNA-205	eDNA-206	eDNA-207	eDNA-208
1042ffb53db165942e8f58b1a5c1cc1e											
7adfb35044222beddd25d5535887890f							9				
f78b7cad11793316ad17f6da1ecb9ec6											
661b0653a6a675f8e7d63c97e61f08fd											
5b8d404061fddbafd74b60fa35b99cc2											
82b75e5ee10a2d01e9cd73c5113cbe41											
0a0d571db32d0a34e760552eca16af9d											

ASV	eDNA-209	eDNA-21	eDNA-210	eDNA-211	eDNA-212	eDNA-213	eDNA-214	eDNA-215	eDNA-216	eDNA-217	eDNA-218
1042ffb53db165942e8f58b1a5c1cc1e											
7adfb35044222beddd25d5535887890f		21			16						
f78b7cad11793316ad17f6da1ecb9ec6											
661b0653a6a675f8e7d63c97e61f08fd											
5b8d404061fddbafd74b60fa35b99cc2		2		1							
82b75e5ee10a2d01e9cd73c5113cbe41											
0a0d571db32d0a34e760552eca16af9d											

ASV	eDNA-219	eDNA-22	eDNA-220	eDNA-221	eDNA-222	eDNA-223	eDNA-224	eDNA-225	eDNA-226	eDNA-227	eDNA-228
1042ffb53db165942e8f58b1a5c1cc1e											
7adfb35044222beddd25d5535887890f		16	6								40
f78b7cad11793316ad17f6da1ecb9ec6											
661b0653a6a675f8e7d63c97e61f08fd											
5b8d404061fddbafd74b60fa35b99cc2		1	1								4
82b75e5ee10a2d01e9cd73c5113cbe41											
0a0d571db32d0a34e760552eca16af9d											

ASV	eDNA-229	eDNA-23	eDNA-230	eDNA-231	eDNA-232	eDNA-233	eDNA-234	eDNA-235	eDNA-236	eDNA-237	eDNA-238
1042ffb53db165942e8f58b1a5c1cc1e											
7adfb35044222beddd25d5535887890f		6							8		
f78b7cad11793316ad17f6da1ecb9ec6											
661b0653a6a675f8e7d63c97e61f08fd											
5b8d404061fddbafd74b60fa35b99cc2									3		
82b75e5ee10a2d01e9cd73c5113cbe41											
0a0d571db32d0a34e760552eca16af9d											

ASV	eDNA-239	eDNA-24	eDNA-240	eDNA-241	eDNA-242	eDNA-243	eDNA-244	eDNA-245	eDNA-246	eDNA-247	eDNA-248
1042ffb53db165942e8f58b1a5c1cc1e											
7adfb35044222beddd25d5535887890f		5					15	1			3
f78b7cad11793316ad17f6da1ecb9ec6											
661b0653a6a675f8e7d63c97e61f08fd											
5b8d404061fddbafd74b60fa35b99cc2							2		1		
82b75e5ee10a2d01e9cd73c5113cbe41											
0a0d571db32d0a34e760552eca16af9d											

ASV	eDNA-249	eDNA-25	eDNA-250	eDNA-251	eDNA-26	eDNA-27	eDNA-28	eDNA-29	eDNA-3	eDNA-30	eDNA-31
1042ffb53db165942e8f58b1a5c1cc1e											
7adfb35044222beddd25d5535887890f	4	5	3					43		40	10
f78b7cad11793316ad17f6da1ecb9ec6											
661b0653a6a675f8e7d63c97e61f08fd											
5b8d404061fddbafd74b60fa35b99cc2	1									2	4
82b75e5ee10a2d01e9cd73c5113cbe41											
0a0d571db32d0a34e760552eca16af9d											

ASV	eDNA-32	eDNA-33	eDNA-34	eDNA-35	eDNA-36	eDNA-37	eDNA-38	eDNA-39	eDNA-4	eDNA-40	eDNA-41
1042ffb53db165942e8f58b1a5c1cc1e											
7adfb35044222beddd25d5535887890f	7	2				40	22	5		7	
f78b7cad11793316ad17f6da1ecb9ec6											
661b0653a6a675f8e7d63c97e61f08fd		49									
5b8d404061fddbafd74b60fa35b99cc2	1					4					1
82b75e5ee10a2d01e9cd73c5113cbe41											
0a0d571db32d0a34e760552eca16af9d										1	

ASV	eDNA-42-MS	eDNA-42	eDNA-43	eDNA-44	eDNA-46	eDNA-47	eDNA-48	eDNA-49	eDNA-5	eDNA-50	eDNA-51
1042ffb53db165942e8f58b1a5c1cc1e	170										
7adfb35044222beddd25d5535887890f	11967				12	3	2	7	13		

f78b7cad11793316ad17f6da1ecb9ec6											
661b0653a6a675f8e7d63c97e61f08fd											
5b8d404061fddbafd74b60fa35b99cc2	1279							1			
82b75e5ee10a2d01e9cd73c5113cbe41	24										
0a0d571db32d0a34e760552eca16af9d	17										

ASV	eDNA-52	eDNA-53	eDNA-54	eDNA-55	eDNA-56-MS	eDNA-56	eDNA-57	eDNA-58	eDNA-59	eDNA-6	eDNA-60
1042ffb53db165942e8f58b1a5c1cc1e					122						
7adfb35044222beddd25d5535887890f		52	36	15	12261	12				14	
f78b7cad11793316ad17f6da1ecb9ec6											
661b0653a6a675f8e7d63c97e61f08fd											
5b8d404061fddbafd74b60fa35b99cc2		3	3		1309						
82b75e5ee10a2d01e9cd73c5113cbe41					10						
0a0d571db32d0a34e760552eca16af9d					13						

ASV	eDNA-61	eDNA-62	eDNA-63	eDNA-64	eDNA-65	eDNA-66	eDNA-67	eDNA-68	eDNA-69-MS	eDNA-69	eDNA-7
1042ffb53db165942e8f58b1a5c1cc1e									74		
7adfb35044222beddd25d5535887890f	16	9	4	7	3				8386	17	2
f78b7cad11793316ad17f6da1ecb9ec6											
661b0653a6a675f8e7d63c97e61f08fd											
5b8d404061fddbafd74b60fa35b99cc2	2	3							1091	2	
82b75e5ee10a2d01e9cd73c5113cbe41									10		
0a0d571db32d0a34e760552eca16af9d					1				12		

ASV	eDNA-70	eDNA-71	eDNA-72	eDNA-73	eDNA-74	eDNA-75	eDNA-76	eDNA-77	eDNA-78	eDNA-79	eDNA-8
1042ffb53db165942e8f58b1a5c1cc1e											
7adfb35044222beddd25d5535887890f	18	1	5					24	21	6	
f78b7cad11793316ad17f6da1ecb9ec6											
661b0653a6a675f8e7d63c97e61f08fd											
5b8d404061fddbafd74b60fa35b99cc2		2						5	4	3	

82b75e5ee10a2d01e9cd73c5113cbe41

0a0d571db32d0a34e760552eca16af9d

ASV	eDNA-80	eDNA-81	eDNA-82	eDNA-83	eDNA-84	eDNA-9	eDNA-M1	eDNA-M2	Total_reads	TaxonomyID
1042ffb53db165942e8f58b1a5c1cc1e							245	244	855	Burhinus
7adfb35044222beddd25d5535887890f	6	4	4	6	8	3	39849	43329	116560	Burhinus
f78b7cad11793316ad17f6da1ecb9ec6									2	Poephila
661b0653a6a675f8e7d63c97e61f08fd									49	Trichoglossus
5b8d404061fddbafd74b60fa35b99cc2							4860	4850	13453	Cacatua
82b75e5ee10a2d01e9cd73c5113cbe41							27	33	104	Cacatua
0a0d571db32d0a34e760552eca16af9d							71	75	190	Aves

ASV	Confidence(%)	Sequence
1042ffb53db165942e8f58b1a5c1cc1e	100	CTCACCTCACTTGAGAGCACATCAGTGAGCACAATAGCCCAACCCGCTAGCAAGACAGGTCAAGGTATAGCCAATGGGAGC
7adfb35044222beddd25d5535887890f	100	GCTCCCATTGGCTATACCTTGACCTGTCTTGCTAGCGGGTTGGGCTATTGTGCTCACTGATGTGCTCTCAAGTGAGGTGAG
f78b7cad11793316ad17f6da1ecb9ec6	89	ATTCCATAGGCTATACCTTGACCTGTCTTATTAGCGTGGTTAGGGCTGTTGCGTCCACTGTTGGGCTTTCAGGGGAGGTGGG
661b0653a6a675f8e7d63c97e61f08fd	81	ACTTCATAGGCTATACCTTGACCTGTCTTGTTAGCGGGTGTGGGTTTTTGGGTCCACTGTTGTGCTCTAATTGGAGGTGGG
5b8d404061fddbafd74b60fa35b99cc2	75	ACTCCATAGGCTATACCTTGACCTGTCTTGTTAGTGGTTGTGGACTATTGGGCTCACTGTTGTGCTTTCATAAAGGTGAG
82b75e5ee10a2d01e9cd73c5113cbe41	75	CTCACCTTTATGAAAGCACAACAGTGAGCCCAATAGTCCACAACCACTAACAAGACAGGTCAAGGTATAGCCTATGGAGT
0a0d571db32d0a34e760552eca16af9d	69	GCTCCCATTGGCTATACCTTGACCTGTCTTGTTAGTGGTTGTGGACTATTGGGCTCACTGTTGTGCTTTCATAAAGGTGAG