Koalas, friends, and foes – the application of airborne eDNA for the biomonitoring of threatened species

Celine Frere[1], Nicola Jackson[2], Alejandro Oliveros Sandino[2], Sarah Ball[2], and Daniel Powell[2]

[1]The University of Queensland
[2]The University of Queensland - Saint Lucia Campus

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Abstract

Perched high up in a Eucalyptus tree, swaying from side to side, lies a sleepy koala unaware of the means spent each year trying to obtain accurate baseline information about its presence. We have thrown all we could at it, from wildlife surveys to night spotting, bioacoustics, detection dogs, and drones equipped with thermal cameras. Yet, whilst critical to its conservation and management efforts, finding a koala remains an ambitious, time-consuming, and costly endeavour often producing insufficient results. However, little did we know that traces of koalas' presence and that of its predators along with other native, domesticated, and invasive species, float in the air and can be detected using metagenomics. This study and despite high levels of co-sampled non-target DNA (e.g. humans and domesticated animals) confirms that koalas, species belonging to the wallaby and possum family and threats such as domestic dogs (a major predator contributing to koala population declines) can successfully be detected by sampling airborne particles. Together, it demonstrates the potential of airborne eDNA for the detection of terrestrial wildlife under natural conditions and presents achievable optimisation steps to increase its field applicability and validity.

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Frère, C.H.[1]; Jackson, N. [1]; Oliveros, A. [1]; Ball, S.J. ; Moreno, J.[1]; Powell, D. [1]

School of Biological Sciences, University of Queensland, St Lucia, QLD, Australia.

Corresponding author: c.frere@uq.edu.au

Abstract

Perched high up in a Eucalyptus tree, swaying from side to side, lies a sleepy koala unaware of the means spent each year trying to obtain accurate baseline information about its presence. We have thrown all we could at it, from wildlife surveys to night spotting, bioacoustics, detection dogs, and drones equipped with thermal cameras. Yet, whilst critical to its conservation and management efforts, finding a koala remains an ambitious, time-consuming, and costly endeavour often producing insufficient results. However, little did we know that traces of koalas' presence and that of its predators along with other native, domesticated, and invasive species, float in the air and can be detected using metagenomics. This study and despite high levels of co-sampled non-target DNA (e.g. humans and domesticated animals) confirms that koalas, species belonging to the wallaby and possum family and threats such as domestic dogs (a major predator contributing to koala population declines) can successfully be detected by sampling airborne particles. Together, it demonstrates the potential of airborne eDNA for the detection of terrestrial wildlife under natural conditions and presents achievable optimisation steps to increase its field applicability and validity.

Introduction

Curbing worldwide wildlife population declines will necessitate the protection of their habitat (Tilman et al. 2017). Aside from political willingness, targeted habitat protection requires robust baseline information about wildlife that use and occupy it. Collating such information, however, remains a challenging and costly endeavour. To tackle these problems, scientists have been working hard developing innovative technologies that improve the cost-effectiveness and scalability of data collection on wildlife assemblages, including employing thermal drones, detection dogs, radars, camera traps and bioacoustics devices (Cristescu et al. 2015, Hagens et al. 2018, Hüppop et al. 2019, Beaver et al. 2020, Law et al. 2020). Whilst these technologies have greatly expanded our capabilities to collect more accurate baseline information on wildlife presence, the collation of data on wildlife assemblages still comes at a significant technological and human cost. To seek to identify the presence of multiple wildlife species, ecologists must deploy a multitude of methods in synchrony, which can include bioacoustics arrays, baited traits, camera traps and human-led fauna transects (Mena et al. 2021). Hence, affordable and scalable technology for the detection and monitoring of wildlife assemblages across the landscape is a goal we should seek to achieve as it would improve our ability to detect and track changes in wildlife biodiversity patterns (e.g. species richness and community composition), identify wildlife species or habitat of high conservation priority, and underpin the development of successful management strategies.

Environmental DNA, known as eDNA is a promising technology to address this issue. eDNA refers to the DNA that is shed into the environment as wildlife move throughout the landscape, leaving previously unbeknownst traces of their presence (Thomsen and Willerslev 2015). Already heavily deployed in aquatic systems for at least two decades, the practicability of utilising eDNA to detect species presence and assemblages is clearly established (Hinz et al. 2022). For example, in 2016, aquatic eDNA technology was first employed as part of environmental impact assessments, demonstrating its capacity to meet regulatory standards and obligations outlined by the International Association for Impact Assessment (Hinz et al. 2022). However, to date, there is no such established terrestrial analogue although recent studies have provided promising results. Terrestrial wildlife's eDNA has, for instance, been detected from samples collected from spider webs, permafrost, blood, snow, soil, honey and aerosol spray runoff (Andersen et al. 2012, Folloni et al. 2012, Schnell et al. 2012, Valentin et al. 2018, Ribani et al. 2020, Gregorič et al. 2022). Two most recent studies have also demonstrated that airborne eDNA can be used to reassemble zoological communities (Clare et al. 2022, Lynggaard et al. 2022). Together, evidence suggest that airborne eDNA could be a promising avenue for the identification of terrestrial wildlife assemblages as it is i) non-invasive, ii) scalable, and iii) comparatively cost-effective given it holds the potential to target species assemblages compared to single species targeted approaches. The feasibility of using airborne eDNA to identify wildlife assemblages under natural conditions remains yet to be tested.

Here, we sought to test the applicability of airborne eDNA particles for the detection of an endangered Australian species, the koala (*Phascolarctos cinereus*), and its co-occurring terrestrial mammalian community in a natural setting. We demonstrate its successful application to detecting koalas as well as other mammalian species in a natural setting and discuss future steps for its continued improvement and optimisation.

Methods

Development and deployment of samplers

Location of sampling was selected for its confirmed long-term koala presence (Cardno 2020). In parallel to the deployment of the airborne eDNA filters, targeted fauna surveys were undertaken to identify koala presence and record their GPS location(s). We designed two types of air filtering sample collection systems which were fitted with sterile cheesecloth for the collection of airborne eDNA. The first air sampling system was composed of a vertical air sampler which allowed for a flow rate of 0.8m/min powered by a waterproof lithium battery pack. This first system was then stationed elevated at approximately 60 cm above ground by securing it to star pickets. The second air sampling system was simply composed of a sterile cheesecloth filter set between sterile stainless-steel plates and attached to the start pickets facing upright in a horizontal

position. These two sampling systems were deployed across 11 sites for 72 hours. All equipment used in the deployment and collection of samples was either purchased gamma sterilised and for single use (e.g., storage tubes) or washed with a bleach solution and autoclaved prior to use (e.g., stainless steel plates). Samples were collected under the animal ethics number 2022/AE000765 from the University of Queensland.

DNA extraction and amplification.

Cheesecloth filters were transferred to sterile 50 ml centrifuge tubes with 10 ml of molecular grade PBS (Gibco) and were rocked gently using a platform shaker for 30 mins at room temperature to dislodge particulate matter. The cheesecloth filters were then removed and the PBS solution centrifuged at 8,000 xg for 10 mins. The supernatant was removed, and the pellet resuspended in 180 µl of Buffer ATL before transfer to a new clean microfuge tube. For lysis, 20 µl of Proteinase K was added and vortexed briefly to mix before being heated at 56°C for 1 hour. DNA was purified using a DNeasy Blood & Tissue kit (Qiagen) following manufacturer's protocol from step 3. DNA was eluted in a total of 100 µl by passing 2 x 50 µl of Buffer AE through the spin column and then stored at -20°C.

Primers selected for this study targeting mitochondrial DNA were previously employed by two recent studies and were validated for use in similar applications (Clare et al. 2022, Lynggaard et al. 2022). DNA was amplified with the primers 16Smam1 forward (5'-CGGTTGGGGTGACCTCGGA-3') and 16Smam2 reverse (5'-GCTGTTATCCC-TAGGGTAACT-3') (Taylor 1996) to produce a fragment of approximately 100 base pairs long (bp). PCR was initially performed in 20 µL reactions consisting of, 10 µl AmpliTaq 360 mastermix (Thermo Scientific); 0.6 µM each of forward and reverse primer and 2 µL DNA template. To reduce the presence of human contamination, the human blocker (5'–3' GCGACCTCGGAGCAGAACCC–spacerC3) as reported by (Vestheim and Jarman 2008a), was included in each reaction. The thermal cycling profile was 95°C for 10 min, followed by 40 cycles of 95°C for 12 s, 59°C for 30 s, and 70°C for 25 s, with a final extension of 72°C for 7 mins. The PCR reactions were visualised on a 1.5% agarose gel alongside non-template controls and koala DNA positive controls. Successfully amplified DNA fragments were selected and subjected to a second round of PCR using primers that included a sequencing tag to enable longer product for sequencing. TSP1_16Smam_forward 5'-TCTACACTCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCGGTTG GGGTGACCTCGGA-3' and TSP2_16Smam_reverse: 5'-GTCTCGTGGGCTCG GAGATGTGTATAAGAGACAGGCTGTTATCCCTAGGGTAACT-3'. For the second-step PCR, conditions were identical to above but for using 1 µl of the PCR product from the first reaction and a lower annealing temperature of 55°C. Samples were sent for amplicon sequencing using the Illumina MiSeq Platform at the Australian Genome Research Facility.

An addition sample of DNA isolated from tissue collected from a skin biopsy an Indo-Pacific bottlenose dolphin (*Tursiops aduncus)* was used as a positive control to check for cross-contamination from demultiplexing and laboratory processing for high-throughput sequencing.

Data analysis

The two airborne eDNA samples collected from each site were combined and treated as one sample to maximise the recovery of cellular material from each site. The raw reads were firstly demultiplexed using QIIME2 (version 2022.8.0) (Estaki et al. 2020) based on the Illumina barcodes, creating separate samples for each. FastQC (version 0.11.9) (*https://www.bioinformatics.babraham.ac.uk/projects/fastqc/*) was used on the demultiplexed reads to assess the overall data quality before and after adapter removal. Adapters were removed using Cutadapt (Martin 2011) (version 2.8-2). Bowtie2 (Langmead and Salzberg 2012) (version 2.2.5) and Samtools (Danecek et al. 2021) (version 1.10) were used to align the reads to a reference human mitochondrial genome sequence (accession number KX456569.1) and identify and remove human/primate-associated sequences from the dataset. In some instances, samples contained 99% human DNA and hence were removed from all further analyses (Supplementary Table 1).

To minimise the introduction of potentially erroneous ASVs, we used our positive control sample (known DNA source; *Tursiops aduncus*) to optimise the filtering and denoising parameters used in the DADA2 package (Callahan et al. 2016) in R (version 1.26.0). We did this by varying the maximum expected errors

from 1 to 2 and the truncation quality score parameters from 2 to 30. Filtering for the maximum expected error of 1 and truncation quality score of 30 significantly decreased the abundance of all reads but did not remove erroneous ASVs. Filtering and denoising parameters were therefore selected to maximise data retention by using default settings: Trimming left of 19 bp for forward reads and 21 bp for reverse reads, truncation length of 130 bp, maximum number of ambiguous bases (N's) of 0, maximum expected errors of 2, truncation quality score of 2, removal of PhiX contamination set to TRUE. DADA2 was then used to remove chimeras from the sequence data using the removeBimeraDenovo function with the pooled method. We then produced two datasets for downstream analyses, one including all forward filtered and denoised reads (to maximise data retention) and the merged forward and reverse filtered and denoised reads. We found that the use of merged reads dramatically reduced the presence of detected contaminant DNA (Figure S1).

The merged, filtered and denoised reads were then used to produce amplicon sequence variants (ASVs) (Table S1), which clusters unique sequence variants based on a 100% sequence similarity. We then used BLAST to match ASVs to mammalian mitochondrial DNA sequences filtered from the NCBI's GenBank non-redundant (nr) database and made species level taxonomic assignments to BLAST hits using the R package taxonomizr (version 0.9.3). Taxonomic assignment was performed using a combination of consensus between the 10 BLAST matches for each ASV and the percentage of identity for each match to produce a trust score. If the percentage identity of one or more matches was higher than the mean of the 10 blast hits, only those higher matches were used to establish a consensus for the assignment of the final taxonomy. The trust score was transformed into a 0-1 score using the formula: trust score = (percentage_identify - 80) / (100 - 80). Only ASVs that returned a mammalian mitochondrial DNA sequence BLAST match, had at least three reads across all samples and did not match to primate mitochondrial sequences were used for subsequent analyses.

A phylogenetic tree was constructed using the ASVs obtained after taxonomic assignment and included relative abundance calculations, normalized by library size. Reference sequences of relevant native and introduced species identified by BLAST matches were also included in the tree construction. These sequences and their accession numbers can be found in Supplementary Table 2. The alignment of ASVs was carried out with the MAAFT (Katoh and Standley 2013) algorithm, and a tree was produced with FastTree (Price et al. 2010) (version 2.1.11). The resulting set of ASVs were manually clustered based on three types of evidence, 1) presence at the same site, 2) the best match to the taxonomic reference, and 3) the normalised abundance. Where multiple, highly similar ([?] 97%) ASVs were present at the same site, the most abundant ASV also had the highest similarity to the taxonomic reference and was therefore chosen as the most accurate representative. This process of manual curation removed the ambiguity of erroneous ASVs from the dataset and aimed to ensure accurate representation of the species present at the sampling sites. A dendrogram was then constructed using this set of curated ASVs and relative abundance was calculated by summing the read counts from the cluster, normalized by library size. Reference sequences of relevant native and introduced animals were included in the tree construction. The tree was then visualized and refined in the Interactive Tree Of Life (iTOL) (Letunic and Bork 2007) tool (version 4.4.0). Additional graphics were applied using Adobe Illustrator.

Results and Discussion

In this study, we have demonstrated that the presence of our targeted threatened species, the koala, and its co-occurring terrestrial mammalian community can be detected from the collection of airborne eDNA under natural conditions. All 11 sampled sites detected the presence of terrestrial mammals (Figure 2) and using a custom taxonomic assignment strategy, we identified the presence of nine taxonomic families, most of which, apart from the Macropodidae and Phalangeridae (kangaroos/wallabies and possums, respectively), were assigned to the species level, including our primary target, the koala. Moreover, we show that airborne eDNA enables the detection of both native and introduced species simultaneously, highlighting the utility of this untargeted sampling approach for the identification of potential biodiversity threats. Dogs, for instance, are a known threat to koalas (Beyer et al. 2018) and were detected from a high abundance of reads across

our 11 sampled sites (Figure 2). We also identified common, but problematic, invasive species including the black rat (*Rattus rattus*) and the hare (*Lepus europaeus*) (Barney et al. 2021, Finlayson et al. 2022). We discuss below how continued methodological optimisation will enable the resolution of some teething problems.

First, and most problematic, is the presence of high level of non-informative co-sampled DNA (e.g. human and domesticated animals). Even with taking the utmost care and using published human DNA blocking primers (Vestheim and Jarman 2008b), we found that many reads were identified as human (Supplementary Table 3). We found that 53% of reads, on average, were lost per sample as they were identified as human DNA and a total of 5 samples were lost as only human DNA was amplified (99% human reads; Supplementary Table 1). While we know that human co-sampling and contamination is a common re-occurring problem when using eDNA (Harper et al. 2019, Leempoel et al. 2020), we also found that, under natural conditions, precious sequencing reads are further lost to non-informative domesticated species, such as cows and horses (Figure 2). This is despite these animals not being identified in faunal transects or prior human led field surveys at the sampling location. The presence of such high abundance of non-informative co-sampled airborne DNA is a challenge we ought to tackle as it likely outcompetes the lower abundance of airborne DNA particles shed by and therefore collected from lower-density species of interest including threatened, endangered, or cryptic elusive species. Our high level of non-informative co-sampled DNA might be one of the reasons why our only sample which detected the presence of koala DNA (Site 11. Figure 2) was the filter located directly beneath a tree occupied by a koala while two additional sampling sites (Site 5 and 2) positioned 50 - 30 meters away from koalas failed to detect their DNA. Whilst this high level of sensitivity in detection range allows us to confirm koala presence with a high degree of confidence, the use of alternative human DNA blocking primers (Boessenkool et al. 2012) coupled with targeted primers to enrich the DNA abundance of low density or low biomass target species prior to Illumina sequencing may help increase our rate and range of detection of low-density, low-biomass species like the koala. A targeted qPCR assay approach will require additional investment in development but when used in concert with an untargeted approach will allow for a more inclusive representation of the occupancy of less abundant species.

Second, we found that our ability to assign species level taxonomic rank was limited by the small target sequence length. While small sequence length is often best when dealing with degraded DNA, a characteristic of eDNA (Beng and Corlett 2020), we identified it imposed some limitations to the taxonomic assignment of our ASVs. For instance, the lack of genetic variation between the mountain and common brushtail possum (1 bp difference) made it difficult for us to assign our ASV to either species with high certainty even though our ASV was 100% similar to the mountain brushtail possum. This is because 1 bp difference between sequences could easily fall within the margin of sequencing error (Stoler and Nekrutenko 2021). Similarly, we were not able to disentangle ASV 19 from a red-neck wallaby or a grey kangaroo because these reference sequences differed by 1 bp. In contrast, we found that some of our ASVs (ASV 14 and ASV 23) contained levels of genetic variation high enough to make taxonomic assignments challenging. The Macropididae ASV #14, for instance, differed from both the Red-Necked and the Swamp Wallaby reference sequence by 5 bp. It, however, phylogenetically clustered with the swamp wallaby (Figure 1) because of a shared conserved region separating them both from the red-necked wallaby. A similar trend was identified for our detected ring-tailed possum ASV (ASV #23) which, while it clustered with our reference ring-tailed possum sequence, nonetheless differed from it by 6 bp. Ecological surveys of our sampling site detected the presence of those three species indicating that, while imperfect at this stage, our taxonomic assignments are likely correct. To continue to improve the robustness of taxonomic assignment, we recommend considering the geographical provenance of reference sequences because of expected geographic patterns of genetic diversity. The ring-tailed possum 16S reference sequence publicly available, for instance, came from an animal located in Western Australia. We therefore propose that prior to deploying airborne eDNA for detection of a suite of target terrestrial species, it is critical to develop a relevant mitochondrial genomic database to ensure accurate taxonomic resolution can be reached. This will include assessing if the targeted sequence region(s) contain sufficient variation to disentangle closely related species and are of geographic relevance to your sampling location. Challenges to obtain material and data from sensitive species groups can be overcome by engagement with

stakeholders, researchers, museums and the community. This is particularly important to the deployment of airborne eDNA technology in natural settings where, unlike zoological facilities (Clare et al. 2022), many closely related species overlap with each other and are only differentiable by a few base pairs resulting in less refined taxonomic resolution or potential misattributed taxonomic assignment.

Last and similar to other eDNA studies (Lusk 2014, Xing et al. 2022), we demonstrate the importance of filtering stringency in the management of DNA contamination (see Figure S1). DNA extracted from a skin biopsy of the Indo-Pacific bottlenose dolphin (*Tursiops aduncus*) was used to estimate and control for DNA contamination in our downstream bioinformatic pipeline, as this species is not found at or near the sampling location. Like many other studies, we did identify a significant amount of DNA cross-contamination which was present in our dolphin sample when we only used forward reads in our bioinformatic pipeline. We, however, found that the merging of ASVs got rid of all DNA cross-contamination which we underline as a necessary step in any future eDNA studies (Supplementary Figure 1).

Conclusion

The sequencing of airborne eDNA using high-throughput technology is a promising tool to survey the terrestrial ecosystem in a cost-effective manner. We demonstrate that it is feasible to use filtered air samples for the recovery of DNA from endangered and hard to find species, such as the koala in its natural environment. Further, we report the detection of 9 taxonomic groups across the surveyed landscape including a range of endemic and introduced species. We propose that in order to increase the representation of species of interest, a targeted approach could be used in concert with a generic assay design with limited additional effort. Nonetheless, this approach has the potential to revolutionise ecosystem wildlife surveys to maximise habitat protection efforts.

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Data Accessibility and Benefit-Sharing.

All ASV produced are included in the supplement information. All raw reads have been uploaded in GeneBank number PRJNA949380.

Authors contribution.

CHF: study design, analyses, data collection, manuscript write up and editing, funding, supervision.

NJ: study design, analyses, data collection, manuscript write up and editing.

OA: analyses, manuscript write up and editing.

SJB: data collection, manuscript editing.

JM: analyses, manuscript write up and editing.

DP: study design, analyses, manuscript write up and editing, supervision.

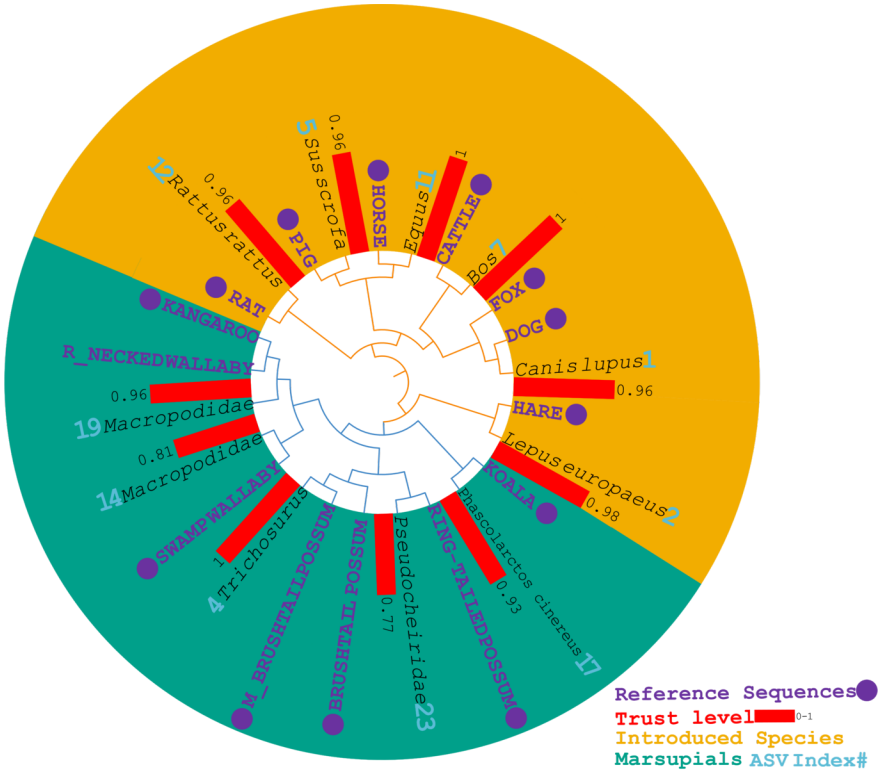

Figure 1. Phylogenetic tree of ASVs after taxonomic assignment at the Birkdale site. Curated ASVs are visualized with a trust score of the taxonomic assignment (represented by red bars next to the taxonomic assignment). Reference sequences are represented by purple dots and the ASV index (blue number) is next to each taxonomic assignment.

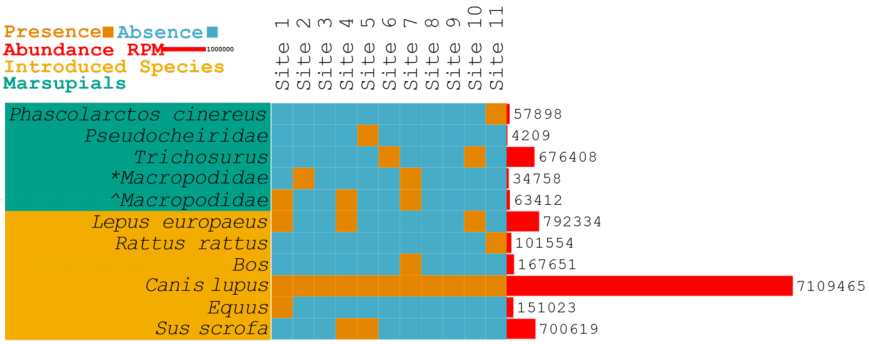

Figure 2. Representation of the Presence/Absence of ASVs after Taxonomic Assignment at Scattered Locations around the Birkdale Site. Curated ASVs are visualized with their total relative abundance in reads per million (represented by red bars), and their presence/absence at 11 different locations around the site (absent = blue cells, present = orange cells). Two macropods, likely a Swamp Wallaby (^ Macropodidae) and a Red-Necked Wallaby (*Macropodidae), were identified.