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Beyond insects: leveraging bulk arthropod samples for vertebrate biodiversity monitoring using invertebrate-derived DNA (iDNA) metabarcoding

Laurence Dugal

laurence.dugal@dbca.wa.gov.au

Department of Biodiversity, Conservation and Attractions

Natasha Harrison

Department of Biodiversity, Conservation and Attractions

Rebekah Hortin

Department of Biodiversity, Conservation and Attractions

Kiara DeLandgrafft

The University of Western Australia

Kristen Fernandes

Department of Biodiversity, Conservation and Attractions

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Abstract

Invertebrate-derived DNA (iDNA) metabarcoding of gut contents from leeches, blow flies or mosquitoes has emerged as a powerful tool to characterise vertebrate communities, but there has been limited exploration on how to adapt this approach to utilise bulk-collected mixed samples of arthropods. Globally, monitoring programs deploy malaise traps to target flying insect communities in a diverse set of environments. These traps often capture suitable iDNA samplers whose associations with fauna can be harnessed to provide information on local vertebrate species assemblages. Here, we utilised bulk arthropod samples collected via malaise traps as part of a forest monitoring program and amplified vertebrate (12S) and mammal (16S) DNA from the samples. We then compared iDNA-derived species lists to detections from camera traps deployed during the same field season. We detected 28 taxa using 12S and 20 taxa using 16S, including mammals, birds, reptiles and amphibians. Focusing on species-level assignments, overall, we detected 17 species from 54 bulk insect samples, including 12 native fauna, two forest pests and three domesticated animals. Over 78% of forest-dwelling fauna detected by iDNA were also detected by cameras, with iDNA yielding three additional detections of small skinks. Despite capturing a much lower diversity than camera trap surveys, iDNA nonetheless showed congruence in detecting common and more cryptic fauna at a site level. By leveraging existing sample collections, this study demonstrates the utility of bulk sample iDNA for monitoring forest vertebrate fauna. With further refinement, this approach provides an opportunity to interrogate existing bulk insect datasets to help complement other forms of fauna survey data in specific settings.

Introduction

Currently, comprehensive data on the total biodiversity of species-rich ecosystems worldwide, including protected areas, remains unavailable (Leponce et al. 2010). Understanding biodiversity by analysing species composition and their interactions is vital for effective conservation and management decisions. However, creating complete taxon inventories is challenging and demands careful, well-considered methodologies. Traditional fauna surveys employ various techniques that vary with the taxonomic group, body size, or ecology of the organisms targeted (Hoffman et al. 2010). This means that to conduct comprehensive biodiversity inventories, multidisciplinary approaches that require a suite of techniques and expertise are needed (Leponce et al. 2010; Mena et al. 2021), which can prove time-consuming, costly, and thus usually limited in terms of scale and taxa recovery (Stem et al. 2005; Deiner et al. 2017). Furthermore, traditional monitoring methodologies such as trapping can be quite invasive, and the potential for trap injuries and deaths should be considered when conducting such surveys (Ali et al. 2018).

Observational methods are thus now widely used to survey certain groups of animals and are considered much less invasive than trapping techniques. Observational methods include direct observations, identification of signs of activity (e.g., tracks, scat, diggings), and camera trapping (Hoffman et al. 2010). The use of camera trapping to survey wildlife has become increasingly more widespread across field-based monitoring programs and applied contexts (Burton et al. 2015). They have major logistical

advantages over conventional trapping methods, with the ability to produce objective, verifiable and archivable datasets, target elusive and cryptic animals, and operate over long periods to allow for higher detection rates (Bruce et al. 2025). Further, with the development of artificial intelligence and machine learning technologies, the effort required to identify species from camera-trap images is gradually decreasing (Bruce et al. 2025; Chalmers et al.. 2023; Leorna and Brinkman 2022). However, cameras still entail significant upfront equipment and installation costs, along with ongoing costs for hosting, maintenance, and processing large image datasets, which limits the number of cameras that can be installed or the locations where they can be deployed (Welbourne et al. 2015; Bruce et al. 2025).

Recently, molecular techniques such as the use of environmental DNA and/or metabarcoding have gained traction for monitoring biodiversity across a range of terrestrial and aquatic environments. In terrestrial settings, environmental DNA has been successfully used to survey a multitude of taxonomic groups, including microbes (Brace et al. 2026), invertebrates (Kirse et al. 2021), pollen (Van Haeften et al. 2024), as well as vertebrates such as birds (Bodawatta et al. 2026), mammals (Newton et al. 2024), reptiles (Santamaria et al. 2024) and bats (Garrett et al. 2023). The technique has been praised as a fast, cost-effective, comprehensive and accurate survey tool for biomonitoring and ecological assessments, strengths which are especially valuable in the age of ongoing biodiversity declines that underscore the need for efficient data collection (Fediajevaite et al. 2021; Thomsen et al. 2024). Beyond eDNA analysis of soil, water or air samples, other sources of DNA such as invertebrate-derived DNA (iDNA) are also being explored for vertebrate monitoring, by targeting the stomach or gut contents of invertebrates that feed on fauna (Calvignac-Spencer et al. 2013b; Broadhurst et al. 2025).

The field of iDNA has established itself as a promising tool in detecting vertebrate species richness (Rodgers et al. 2017; Gogarten et al. 2019). It has been used to examine ecological patterns such as assessing species diversity across disturbance gradients (Drinkwater et al. 2020), compare community composition across seasons (Chivas et al. 2025), infer occupancy estimates (Abrams et al. 2019) and evaluate the efficacy of protected areas (Ji et al. 2022). A range of iDNA sampler species have been tested, including mosquitoes (Chivas et al. 2025), blow flies (Smith et al. 2026), sandflies (Massey et al. 2022), leeches (Drinkwater et al. 2020), biting midges (Cutajar and Rowley 2020), carrion flies (Fernandes et al. 2023) and dung beetles (Saranholi et al. 2024). However, studies typically deploy traps to sample one specific vertebrate-feeding insect for the distinct purpose of vertebrate monitoring. One exception is a study by Lynggaard et al. (2019), which utilised bulk arthropod samples collected using malaise and pitfall traps in tropical forests, and demonstrated successful amplification of vertebrate DNA from mixed insect samples. However, since the Lynggaard et al. (2019) paper, there has been no further application of this technique in any other ecosystem. Insect surveys using bulk-collection techniques are generally standardised and commonplace (Montgomery et al. 2021). Thus, there is significant value for opportunistic interrogation of these samples to also investigate vertebrate diversity, especially if DNA extractions have already been generated for other metabarcoding applications.

Here, we examined the use of bulk arthropod iDNA for monitoring terrestrial vertebrates in Eucalyptus forest ecosystems. We utilised bulk arthropod samples collected as part of a yearly forest monitoring

program in southwestern Australia and targeted the vertebrate DNA within the mixed insect samples. To benchmark iDNA detections against known local fauna records, we compared species-level vertebrate fauna detected via malaise traps over a two-week period to contemporary records of fauna diversity observed via camera traps deployed at the same sites over a 6-week period during the same Austral spring field season.

Materials and Methods

Camera traps

Data were collected as part of an ongoing forest monitoring program designed to track forest health in southwestern Australia, with 100 forest sites located in different Eucalyptus forest ecosystems, including Jarrah (*Eucalyptus marginata*), Wandoo (*E. wandoo*), Karri (*E. diversicolor*) and Tingle (*E. jacksonii/E. guilfoylei/E. brevistylis*) (DBCA 2025). For this pilot study, we focused on a subset of 54 sites (Fig. 1). At each site, two motion sensor cameras (Reconyx HP4K) were deployed for a minimum of 6 weeks in Austral spring 2025 (September to November). Cameras were attached to trees or stakes, approximately 20-30cm above the ground, facing outward and programmed to take three images per trigger with the “Long Range” focal length and no quiet period. This particular design targeted small- and medium-sized terrestrial mammals. At a subset of these 54 sites (n = 14), an additional outward facing camera, a downward facing camera and an arboreal camera were also deployed in the same period to expand the taxa detected. Downward facing cameras were designed to target reptiles. They were attached to stakes 1.0-1.5m above the ground, facing onto a cork tile, and were programmed to take three images per trigger with “High Frequency” focal length and no quiet period, as well as take timelapse images every 3 minutes from 09:00 until 17:00 each day of deployment. The arboreal cameras were designed to target arboreal mammals and were attached to a platform fixed to a tree using 90° shelf brackets with the camera facing toward the trunk (see Cowan et al. 2024 for more details). These cameras were programmed to take three images per trigger with no quiet period, using the “Hyperfire Legacy” focal length. While there was variation in the number and duration of camera deployment at each site (see Table S1 for more details), the cameras provide a reliable snapshot of contemporary fauna occurrence at each site against which the iDNA sampling can be benchmarked. We used this approach as the camera data is more temporally accurate than using historical records given that the data were collected at the same site during the same season.

Malaise traps

Bulk arthropod samples were also collected as part of the same forest monitoring program (DBCA 2025). Flying insect samples were collected using malaise traps (EZ Malaise Trap II Townes Style) during September to November 2025. One malaise trap was deployed at each site and left for approximately two weeks (range of 14–19 days). Bulk insects were captured in 100% molecular grade ethanol, and collection bottles were wrapped in aluminium foil to prevent significant evaporation of the ethanol during

collection windows. Following the collection of sample bottles from the field, bulk insect samples were topped up with fresh 100% ethanol and stored at -20°C until further processing.

DNA extraction and metabarcoding

Ethanol was removed from bulk insects by sieving the samples using plastic sieves that were sterilised between uses with a 30-minute soak in 10% bleach solution and 30-minute exposure to UV radiation. Bulk samples were left at room temperature for a few hours to evaporate the remaining ethanol to ensure no carry-over during DNA extraction. Bulk insects were then transferred to 50 mL Falcon tubes (Eppendorf) containing four steel balls. For organisms larger than a honeybee, only 1–2 legs were used for homogenisation (van der Heyde et al. 2022). Approximately 10–20 mL of molecular-grade UltraPure water was added to each tube to facilitate homogenisation of the dry insect samples. Homogenisation of samples was conducted using a TissueLyser (Qiagen) for 2 minutes in 30-second increments at 30 Hz. DNA extractions were carried out using a DNeasy Blood and Tissue kit (Qiagen) following the manufacturer's protocol, except for the following modifications: 400 µL of the insect homogenate was digested overnight at 56°C with 360 µL of ATL buffer and 40 µL of Proteinase K solution. Then, 400 uL of this digested material was purified and extracted using an automated Qiacube (Qiagen), with a final elution volume of 100 µL. Extractions were carried out over several days, and an extraction blank (EB) was included on each day of extractions.

Two vertebrate-specific metabarcoding primer sets were used to amplify iDNA of mammals and vertebrates from the bulk insect samples. A ~ 98bp fragment of the 12S gene was amplified using the 12Sv5 primers (12Sv5F: 5'-TAGAACAGGCTCCTCTAG-3'; 12Sv5R: 5'-TTAGATACCCCACTATGC-3') (Riaz et al. 2011) to target vertebrates more broadly and a ~ 90bp fragment of the 16S gene was amplified using the 16Smam primers (16Smam1-F: 5'-CGGTTGGGGTGACCTCGGA-3'; 16Smam2-R: 5'-GCTGTTATCCCTAGGGTAACT-3') (Taylor 1996) to target mammals specifically. These primers have been shown to be effective at recovering Australian native mammals (Campbell et al. 2024) and have also been successfully used in eDNA studies targeting terrestrial vertebrates in the region (e.g. Ryan et al. 2022; Newton et al. 2024), including iDNA studies (Fernandes et al. 2023; 2024). PCR reactions were made up to 25 µL containing 12.5 µL GoTaq G2 HotStart master mix (Promega), 1.2 µL each of forward and reverse primers (10 µM) containing the Nextera indexes required for Illumina sequencing, 8.1 µL of nuclease-free water, and 2 µL of DNA. Thermocycler conditions were as follows: 95°C for 5 minutes, followed by 45 cycles at 95°C for 30 seconds, annealing at 52°C (12S) or 55°C (16S) for 30 seconds, extension at 72°C for 45 seconds followed by a final extension at 72°C for 10 minutes. Each sample was run in duplicate during PCR and blended before library preparation. A no-template control (NTC) and a positive control (POS) were included on every PCR plate. Positive controls used were goat and fox DNA. Amplification was verified on a 1.5% agarose gel. Laboratory controls (EB or NTC) were not sequenced if they did not show a band on the gel. Second-stage library preparation and sequencing were performed at the Ramaciotti Centre for Genomics at the University of New South Wales in Sydney, Australia, using a MiSeq i100 Plus 5M 2x150bp cycle run (Illumina).

Bioinformatics and data analyses

Sequence data were processed separately for each primer set. Sequences were obtained demultiplexed from the sequencing provider and processed using the DADA2 pipeline (v1.29.0; Callahan et al. 2016) in R Studio (v2023.06.1). Amplicon sequence variants (ASVs) were assigned taxonomy using the NCBI GenBank database and the Basic Local Alignment Search Tool (BLASTn) on a high-performance cluster computer (Pawsey Setonix Supercomputer, Pawsey Research Centre). BLASTn settings were set at qcov 95, perc_identity 95, evalue 0.05, max_target_seqs 10. We used the lowest common ancestor (LCA) script in the eDNAFlow pipeline to assign taxonomy based on the top 10 BLAST hits (Mousavi-Derazmahalleh et al. 2021). The LCA output was manually verified to validate assignments, and BLAST results were checked to ensure that the detected species were accurate for the study location. For taxa where only one species occurs in the study area, but where the LCA script collapsed the ASV to a higher taxonomic level, the family or genus-level assignment was manually reassigned to that species. Taxa rearranged in this way included Tachyglossidae (*Tachyglossus aculeatus*) in the 12S dataset and *Canis* (*Canis lupus familiaris*), *Notamacropus* (*Notamacropus irma*), Pseudocheiridae/*Pseudocheirus* (*Pseudocheirus occidentalis*), *Capra* (*Capra hircus*), *Ovis* (*Ovis aries*) in the 16S dataset. However, species-level assignments were only accepted if they had an identity match > 98% to a NCBI reference sequence. Taxonomic files were filtered to remove human and non-target taxa such as fish and insects. Reads from ASVs with the same taxonomic assignment were combined across samples and detections were combined across the 12S and 16S datasets. To allow comparisons with the camera trap data, only species-level assignments were retained for further analyses. The final counts matrix was transformed to a binary matrix. A Venn diagram comparing species diversity detected across methods was generated using eulerr (v8.1.0; Micallef and Rodgers 2014; Wilkinson 2012). To compare species diversity detected across sites between methods, we performed a Wilcoxon signed-rank test. This non-parametric test was selected because the distribution of within-site differences violated the normality assumption, as indicated by a Shapiro test. To examine species richness achieved with the iDNA sampling effort, species accumulation and coverage curves were computed using iNEXT (v3.0.2; Hsieh et al. 2016) and all other plots were generated using ggplot2 (v4.0.3; Wickham 2016).

Results

Sequencing results

A total of 2,168,000 sequences and 2,957,291 sequences were obtained using 12S and 16S, respectively. Following DADA2 filtering, a total of 1,325,973 sequences remained for 12S and 1,062,830 sequences for 16S. On average, we recovered 23,262 (± 6,659) reads per sample using 12S and 18,646 (± 12,586) reads per sample using 16S.

Metabarcoding using the 12S primer yielded 28 unique taxa comprising 19 species-level, three genus-level and six family-level assignments. Detections were primarily Mammalia (57.1%), followed by Aves (25%), Lepidosauria (14.3%) and Amphibia (3.6%). The majority of species-level hits consisted of

mammals (14/16 taxa) and reptiles (3/4 taxa), however, taxonomic resolution within birds was poor, where the seven taxa consisted of two species-level hits, two genus-level hits and three family-level hits. The only amphibian record was also detected at family level only (Table S2). *Bos taurus* (cow) was detected in the extraction blank and in four iDNA samples and was thus removed from the dataset. No taxa were found in the NTC. The positive control contained fox (*Vulpes vulpes*) as expected, as well as dog (*Canis lupus familiaris*). Fox was not recovered in the iDNA samples, but dog was also detected in one sample. These hits were discarded from the dataset. Following this data cleaning and considering only species-level detections from the bulk insect samples, the 12S primer set detected a total of 16 species.

Metabarcoding using the 16S primer yielded 20 taxa comprising of 17 species-level assignments and three family-level assignments. Detections were predominantly Mammalia (90%), followed by Lepidosauria (10%). The majority of mammal detections consisted of species-level hits (16/18 taxa), whereas the two reptile detections comprised both a species and family-level assignment (Table S3). *Bos taurus* (cow) was detected in the extraction blank and in two iDNA samples and was thus removed from the dataset. No taxa were found in the NTC. In the positive control, we detected goat (*Capra hircus*) and fox as expected, but also several other taxa: sheep (*Ovis aries*), dog, rabbit (*Oryctolagus cuniculus*), Bovidae sp. and Canidae sp. All but Bovidae sp. and Canidae sp. were also detected in iDNA samples (three for sheep, three for dog, one for rabbit). These ASVs were discarded from the dataset. The positive control also contained a single read belonging to western grey kangaroo (*Macropus fuliginosus*), but as this represented a minimal read number compared to the range detected in the other samples (449 – 36,770 reads, mean = 7,962), we believe this detection to be biologically valid, and it was retained in the dataset. Following this data cleaning and considering only species-level detections present in the bulk insect samples, the 16S dataset detected a total of 11 vertebrate species. Most of these species were also detected in the 12S dataset, except for the western ringtail possum (*Pseudocheirus occidentalis*), which was only detected with the 16S assay.

Diversity detected via iDNA

Combining detections across the two assays resulted in a final list of 17 vertebrate species detected from 54 malaise trap samples; 12 mammals, three reptiles and two bird species. Most species detected were native animals (70.6%), but we also detected forest pest species (e.g. pig, rabbit) as well as farm animals likely from nearby agricultural land (e.g. chicken, sheep, horse). Vertebrate species were detected in 41 of the 54 (~ 75.9%) bulk arthropod samples, with one to six species detected in each sample (corresponding to each site visited). The most commonly detected species was the western grey kangaroo, occurring in 31/41 samples (75.6%), followed by the western brush wallaby (*Macropus irma*; 22/41 samples, 53.6%) and the common brushtail possum (*Trichosurus vulpecula*; 14/41 samples, 34.1%). Short-beaked echidna (*Tachyglossus aculeatus*) and pig (*Sus scrofa*) were detected in six and five samples, respectively, whilst the remainder of the fauna were detected in three or fewer samples (Table S4).

The species accumulation curve indicated that our sampling effort fell short of capturing the estimated species richness of 34, and substantially more sampling was required (Fig. 2A). Had all 100 samples collected in the forest monitoring program been processed, this would have resulted in only a marginal increase in species detections, with closer to 350 samples are required to reach the estimated diversity asymptote. However, sample completeness (sample coverage), or the proportion of total assemblage occurrences represented by the detected species, was high (0.941) (Fig. 2B). This suggests that the current sampling effort captured the dominant species that make up 94.1% of all vertebrate detections, but was less efficient at capturing the rare, low abundance species, preventing the curve from reaching saturation.

Figure 2 Species accumulation and sample completeness curves showing vertebrate species detection using iDNA with the current sampling effort of 54 samples. Panel A) indicates species diversity (n = 17) detected and panel B) shows sample coverage obtained across samples. Full lines indicated interpolated data, and dotted lines indicates extrapolated data. Shaded areas indicate the 95% confidence intervals

Comparison with camera trap data

Camera traps detected a total of 62 species across 54 sites, including 20 mammals, 36 birds and six reptiles (Table S5). A range of four to 23 vertebrate species were detected per site. To ensure adequate comparisons with the camera trap data, we focused only on the 14 forest-dwelling fauna detected via iDNA and therefore removed the three farm animals from further comparisons. Approximately 78.5% (11/14) of iDNA-derived vertebrate species were also detected using camera traps (Fig. 3). All species detected by both methods were mammals, except for one bird, the western thornbill (*Acanthiza inornata*). Species present in both datasets included common fauna such as kangaroos and wallabies, as well as more cryptic marsupials such as quokka (*Setonix brachyurus*). Unique iDNA detections included three species of small skinks, whereas camera traps uniquely detected high bird diversity, common invasive pests (e.g., *Felis catus* (cat), fox, *Rattus rattus* (rat)), and important native fauna of conservation interest (e.g., *Cercartetus concinnus* (western pygmy possum), *Dasyurus geoffroii* (chuditch), (*Sminthopsis fuliginosa fuliginosa* (grey-bellied dunnart), *Varanus rosenbergi* (heath goanna) (Table S5).

Species diversity detected per site differed significantly between survey methods, with camera traps detecting higher diversity than iDNA at all sites (Wilcoxon test, $V = 0$, $p < 0.001$). In 19/54 cases, combining datasets increased the number of species detected (Fig. 4A). There was widespread congruence in the detection of common species across sites, for example, 90.3% of iDNA detections of western grey kangaroos were validated by camera-trap data (Fig. 4B). Likewise, we observed matches for western brush wallabies at 77.3% of sites and for common brushtail possums at 71.4% of sites. On the other hand, we also found notable co-detections of more cryptic fauna such as quokkas and woylies (*Bettongia penicillata*), in which at all three sites where these species were detected via iDNA, they were also observed via camera traps (Fig. 4B).

Additionally, iDNA detections supplemented site-level fauna records by detecting species not captured via camera traps (Fig. 4B). Kangaroo was detected at an additional four sites, wallaby at five extra sites, brushtail possum at four extra sites, echidna at two, and lastly, quenda (*Isoodon fusciventer*) and western thornbill at one extra site each (Fig. 4B). iDNA records also yielded additional detections of pest species, including three extra site-level observations of pigs and one of rabbits (Fig. 4B).

Discussion

This study showed that vertebrate DNA can be incidentally recovered from bulk arthropod samples collected using malaise traps, highlighting the utility of mixed-sample iDNA for gathering information on local fauna diversity. Whilst the majority of vertebrate species detected were forest-dwelling species, we also detected some farm animals from nearby agricultural land, emphasising the uncertainty of the spatial scale of vertebrate signals detected via flying insect iDNA. Despite capturing a significantly lower diversity than camera traps, this iDNA survey nonetheless showed the potential of utilising already collected samples for alternative purposes, therefore maximising the biodiversity information gained from routine monitoring. This is especially valuable when resources are limited to undertake comprehensive biodiversity surveys at a large spatial scale.

Vertebrate diversity detected via iDNA

Combining detections across the two primer sets used, we detected 17 vertebrate species using iDNA metabarcoding. The majority of detections were forest dwelling fauna, including some well-known pest species that are frequently observed in these areas. However, we also detected three farm animals (chicken, horse and sheep), indicating that proximity to other environments can generate non-target detections. These three farm animals were detected from five sites (out of the 54), despite relative proximity of agricultural land at many of the sites surveyed, indicating that these non-target signals may be relatively sparse and could be dominated by DNA signatures of local fauna in most cases. Likewise, Saranholi et al. (2023) detected some domestic and non-enclosed species living freely inside or outside of the zoo where they conducted their study, and Smith et al. (2026) detected domestic species in a fragmented forest landscape. A number of studies have attempted to quantify the localisation of iDNA signals to better understand the spatial extent of iDNA detections (Saranholi et al. 2023). For flies and mosquitoes, there is large variability in the distance from source DNA recorded, ranging from highly localised signals of a few hundred metres (Saranholi et al. 2023) to detections up to a few kilometres away (Lee et al. 2023; Fernandes et al. 2024). However, when comparing carrion fly iDNA from conservation reserves to road edges, Fernandes et al. (2023) found differences in species diversity and composition, reflecting their predictions in species distributions. This suggests geographical limits to the extent a vertebrate species' iDNA signal can be dispersed (Fernandes et al. 2023). In our case, because bulk arthropod samples contain a variety of different iDNA samplers with varying feeding strategies and dispersal capabilities, geographical extent cannot be readily quantified, and iDNA signals may encompass detections from species occurring in close proximity to the malaise trap, as well as species

present in nearby surrounding areas. We therefore urge caution when interpreting the spatial and temporal resolution of bulk arthropod-derived detections (Lynggaard et al. 2019).

Comparison of species richness with camera traps

Comparing iDNA detections to contemporary fauna records, we found that iDNA consistently detected a lower species richness than camera traps at a site level, as well as overall across the landscape, with a limited overlap in the species detected by both methods. Some notable species missed by iDNA metabarcoding include problematic invasive predators such as foxes and cats, although these species were also sparsely detected by cameras (6 sites each). We also failed to detect several native fauna species (beyond avian diversity) using iDNA, including arboreal mammals such as western pygmy possums, small marsupials (mardo (*Antechinus flavipes*), dunnart and chuditch) and reptiles (e.g., goanna, bobtail (*Tiliqua rugosa*)). One potential explanation for the discrepancies between the iDNA and camera trap datasets is that the vertebrate-feeding invertebrates collected did not feed on all available hosts within the forest sites sampled. Studies on blood meal analyses suggest that some invertebrates do not feed solely opportunistically and therefore exhibit a degree of host preference, and overall, this is poorly understood across most blood-feeding insects (Lyimo and Ferguson 2009; Calvignac-Spencer et al. 2013b; Hanya et al. 2019). Host preference and feeding ecology will therefore likely impact the inventory of vertebrate species detected via iDNA and may not always reflect the local diversity (Calvignac-Spencer et al. 2013b; Hanya et al. 2019). Another potential cause for the limited overlap in species detections across methods are the different deployment periods of data collection. While both survey types were conducted in Spring 2025, camera traps were deployed for approximately six weeks (with some sites receiving additional sampling effort), whereas malaise traps collected flying insects over approximately two weeks. iDNA-derived species lists may therefore represent only a subset of local diversity, and increased species recovery may have been achieved if malaise traps were deployed for the whole duration of the camera trapping period. Despite this variable sampling effort between the two survey methods, we nonetheless used the camera trap data as the most contemporary fauna record dataset against which to compare iDNA detections.

Lastly, iDNA faces the same limitations as other metabarcoding approaches, and incomplete reference databases may have hindered the taxonomic resolution achievable in this study (Rodgers et al. 2017; Saranholi et al. 2023). We detected 28 and 20 taxa using 12S and 16S, respectively, however, we only retained species-level taxonomic hits to allow comparisons with species lists detected from camera traps. This was particularly evident in the 12S dataset, where nine taxa could not be resolved below family or genus levels (five birds, two mammals, one reptile and one amphibian). As genomic resources continue to expand, we anticipate that more comprehensive species-level inventories will be more readily achieved with DNA metabarcoding. Adding to this, increasing sample replicates per site (DNA extractions) to better capture the range of iDNA samplers (and their vertebrate hosts) within each bulk sample, as well as elongating the malaise trap deployment windows, may help increase the vertebrate biodiversity recovered using bulk arthropod samples.

Overall, studies comparing iDNA to traditional survey methods such as camera trapping show great complementarity between approaches, with iDNA allowing for the capture of additional species, or approaching similar species richness with lower sampling effort (e.g., Gogarten et al. 2019; Massey et al. 2022). In our case, bulk iDNA collected from Eucalyptus forests yielded lower diversity than camera trapping, but supplemented fauna records with three additional species of skinks, as well as site-level observations of a range of fauna. We also observed that both common, abundant large-bodied species (e.g. kangaroo and wallaby), as well as smaller more cryptic species (e.g. quokka, quenda) could be readily detected via bulk insect iDNA metabarcoding. It is worth noting that the iDNA method was capable of detecting species not well targeted by camera traps, or those hard to effectively identify to species level from camera trap imagery (e.g. skinks). In comparison, in a study applying carrion fly iDNA to a tropical mammal community, iDNA detected a higher number of species than cameras, but the technique also failed to detect some abundant species commonly observed visually (Rodgers et al. 2017). Massey et al. (2022) showed that in the southern Amazon, carrion fly and sandfly iDNA, but not mosquito iDNA, detected more species than cameras, and that overall, iDNA-described diversity better captured arboreal and smaller-bodied mammals. On the other hand, cameras resulted in higher species richness than leech iDNA in Malaysian Borneo, but authors suggested that increasing leech collection effort would yield comparable results (Abrams et al. 2019). Across tropical Africa, fly-derived iDNA and camera traps detected a similar number of species, with a limited overlap in detections at a site level highlighting the complementarity of multidisciplinary surveys (Gogarten et al. 2019).

Our iDNA survey, however, produced low detectability of amphibians, reptiles and birds. For example, we only detected one bird species using iDNA compared to the 36 species detected via camera trapping. Beyond the potential impact of the shorter sampling period of iDNA compared to camera traps, this discrepancy may be a result of the feeding preferences of the iDNA samplers, or because the metabarcoding primers used are not optimised for this taxonomic group. Other iDNA studies also note a higher proportion of mammals detected compared to other taxonomic groups (Calvignac-Spencer et al. 2013a; Fernandes et al. 2024), however birds have also been well recovered in other studies (Lynggaard et al. 2019; Chivas et al. 2025). The two primers employed here are commonly used in the iDNA literature, with the 16S primers designed to target mammals, whilst the 12S can amplify vertebrates more broadly (Taylor 1996; Riaz et al. 2011). Incorporating a bird-specific metabarcoding assay in future iDNA studies may help yield a more comprehensive inventory of avian diversity (e.g., Epp et al. 2012; Ushio et al. 2018; Newton et al. 2023; Liang et al. 2026; Thalinger et al. 2026). Interestingly, in a study on leech iDNA, Ji et al. (2022) utilised the same two assays as our study but detected 86 vertebrate species including a high number of amphibians, birds, and mammals, as well as some squamates. Their sampling effort was, however, substantial, with over 30,000 leeches collected across a 677 km2 reserve in China (Ji et al. 2022). Alternatively, iDNA approaches that specifically target frog-biting midges may be more effective at capturing amphibian diversity than bulk arthropod iDNA (Cutajar and Rowley 2020). The type of iDNA sampler selected will have an impact on the taxonomy detected (Massey et al. 2022; Saranholi et al. 2023) and care should be taken to select the appropriate method for each study. By providing a non-targeted approach, bulk arthropod iDNA may be better suited at capturing vertebrate

communities broadly, whereas specific iDNA samplers applied with a particular goal may be better suited for detecting particular taxonomic groups.

Methodological considerations

We detected non-human vertebrate signals in ~ 76% of bulk arthropod samples processed, which is in line with the 56.9–90.6% detection rate reported by the only other bulk arthropod iDNA study (Lynggaard et al. 2019). This shows that despite the untargeted nature of sample collection, these mixed samples still contain informative data on local fauna. However, we detected only a small proportion of the known vertebrate diversity from the southwestern Australian forests sampled, indicating that further refinement is needed to expand species recovery from bulk iDNA techniques. The bulk arthropod samples collected via malaise traps contain a variety of flying insects, many of which are not useful iDNA samplers as they do not feed on vertebrates. It is possible that the vertebrate DNA contained in the mixed samples is largely overwhelmed by the total invertebrate DNA present, therefore limiting our ability to adequately capture local terrestrial vertebrate species (Lynggaard et al. 2019). Increasing the number of DNA extraction replicates for each mixed sample may help generate more comprehensive biodiversity inventories and future studies should examine if increases in fauna recovery are attained with each additional extraction replicate. Although we examined species accumulation curves and sampling effort, we could only do so across all forest sites and understanding where the plateauing in species richness occurs at an individual sample level would be beneficial. This would help more thoroughly evaluate the utility of processing existing bulk arthropod sample collections for vertebrate species monitoring. It should also be noted that we performed two PCR replicates per sample for each metabarcoding assay and pooled these for sequencing. Incorporating additional PCR replicates could also improve our ability to detect low levels of template DNA in the sample (Alberdi et al. 2018). This has been observed to some extent in the bulk iDNA study by Lynggaard et al. (2019) study, where, despite not specifically assessing how PCR replication influenced fauna detection rates, they nonetheless remarked that several taxa were only detected due to their PCR pooling strategy and might otherwise have been missed.

Future directions

Our findings contribute to growing evidence of the utility of invertebrate-derived DNA metabarcoding for surveying terrestrial fauna and represent only the second study utilising bulk arthropod samples for this purpose. We argue that existing sample collections from global malaise trap monitoring programs, such as Lifeplan (https://www.helsinki.fi/en/projects/lifeplan) or BIOSCAN (Hobern 2021), may serve as useful long-term biobanks that incidentally captured local diversity beyond the invertebrates they were targeting. Additional interrogation of these samples could provide useful information on vertebrate communities, and, especially in global initiatives, this could span large time scales across many ecosystems, including providing historical baselines for comparison with new datasets. However, this is subject to the caveat that vertebrate detection from samples not previously collected for DNA metabarcoding may be ineffective. This is because not only does arthropod DNA undergo degradation within malaise traps (Krehenwinkel et al. 2018), but the degradation rates of vertebrate DNA in bulk samples are also not well understood (Lynggaard et al. 2019). Investigation of the appropriate

methodologies for collections, treatment and storage of samples that protect from DNA degradation in bulk insect samples represents a promising avenue for future research.

Declarations

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Competing Interests

The authors have no relevant financial or non-financial interests to disclose.

Author Contribution

LD and KF designed the study. Data collection was performed by LD, NH, KDL and RH. Data analysis was performed by LD, NH and KDL. The first draft of the manuscript was written by LD and all authors read and approved the final manuscript.

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Data Availability

Files corresponding to raw sequence data, DADA2 outputs for 12S and 16S and metadata were deposited on the Zenodo.org repository and are available at the following URL (https://doi.org/10.5281/zenodo.21767173). All other data supporting the findings of this study are available within the paper and its Supplementary Information.

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Figures

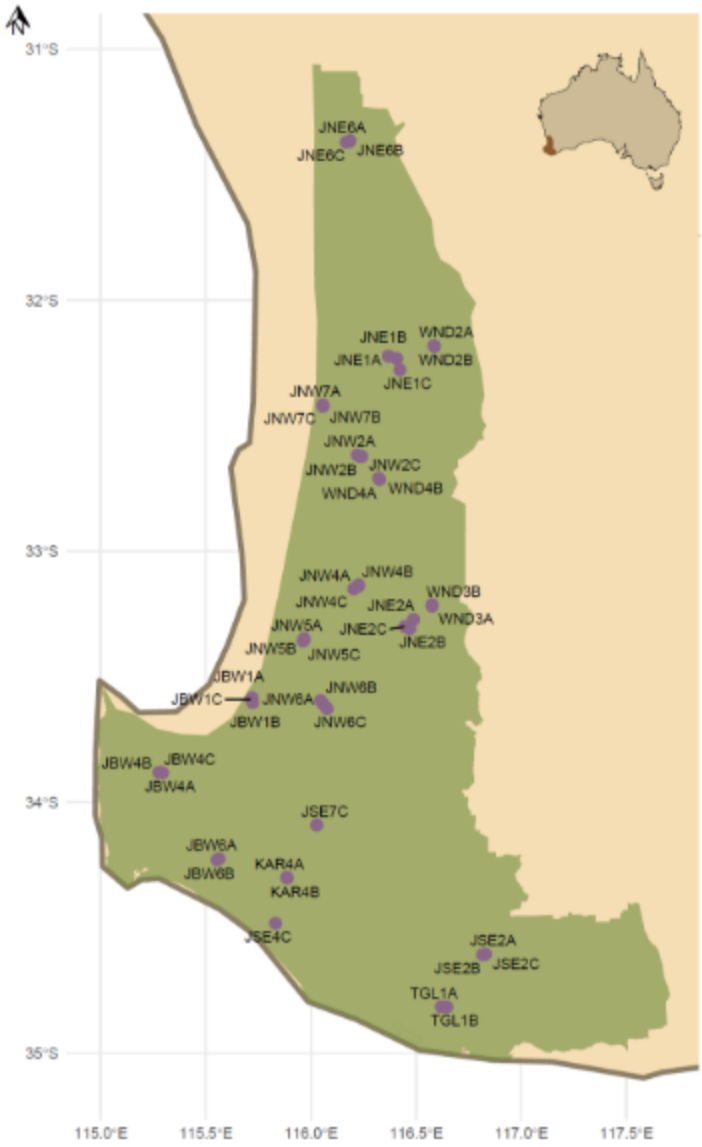


Figure 1

Map of the study sites (n = 54) in southwestern Australia. The green polygon represents the approximate area containing remnant native forest

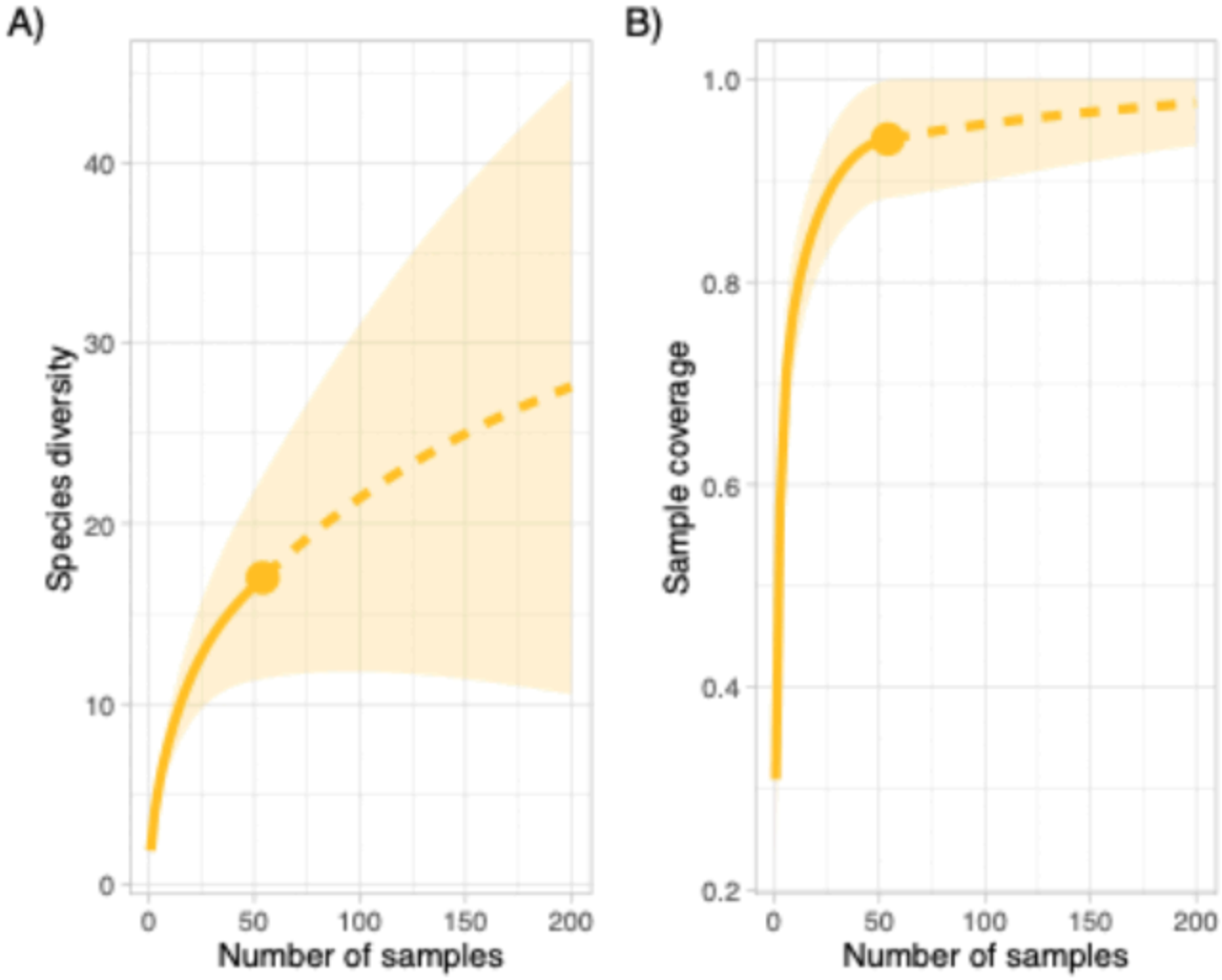


Figure 2

Species accumulation and sample completeness curves showing vertebrate species detection using iDNA with the current sampling effort of 54 samples. Panel A) indicates species diversity (n = 17) detected and panel B) shows sample coverage obtained across samples. Full lines indicated interpolated data, and dotted lines indicates extrapolated data. Shaded areas indicate the 95% confidence intervals

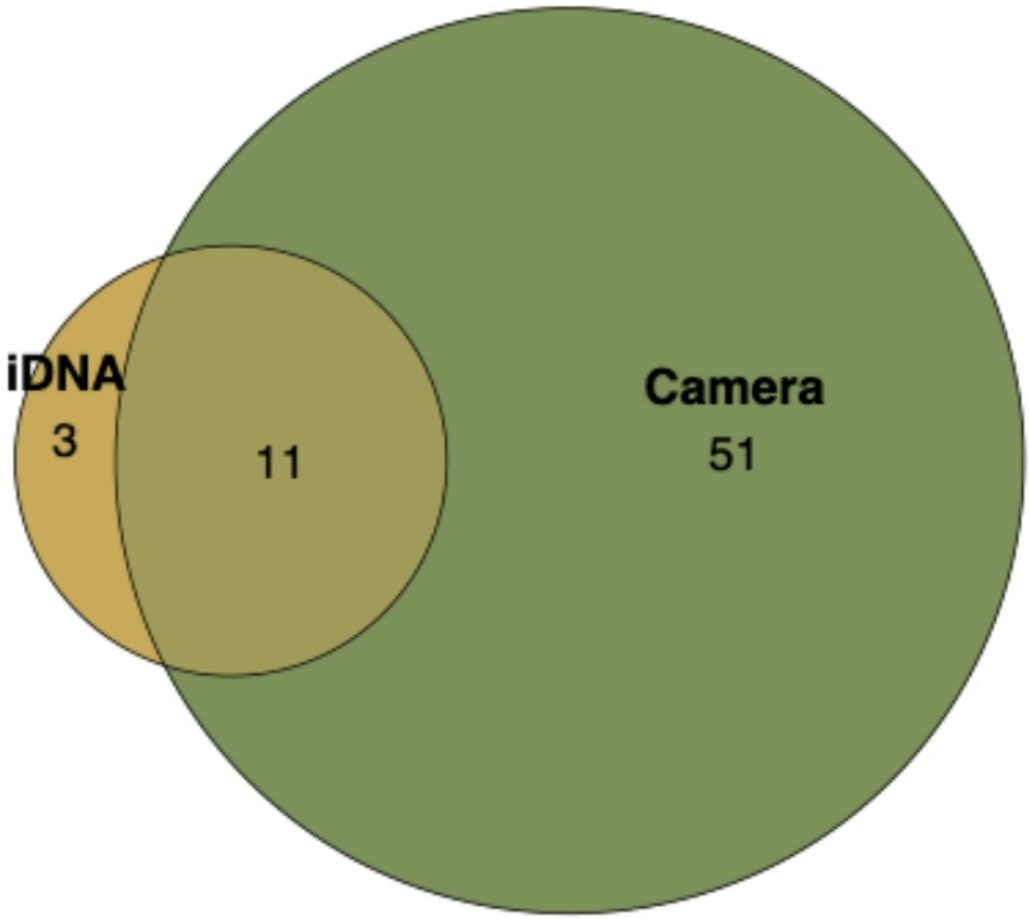


Figure 3

Venn diagram of forest-dwelling vertebrate species detected by iDNA and camera traps at 54 forest sites throughout southwestern Australia

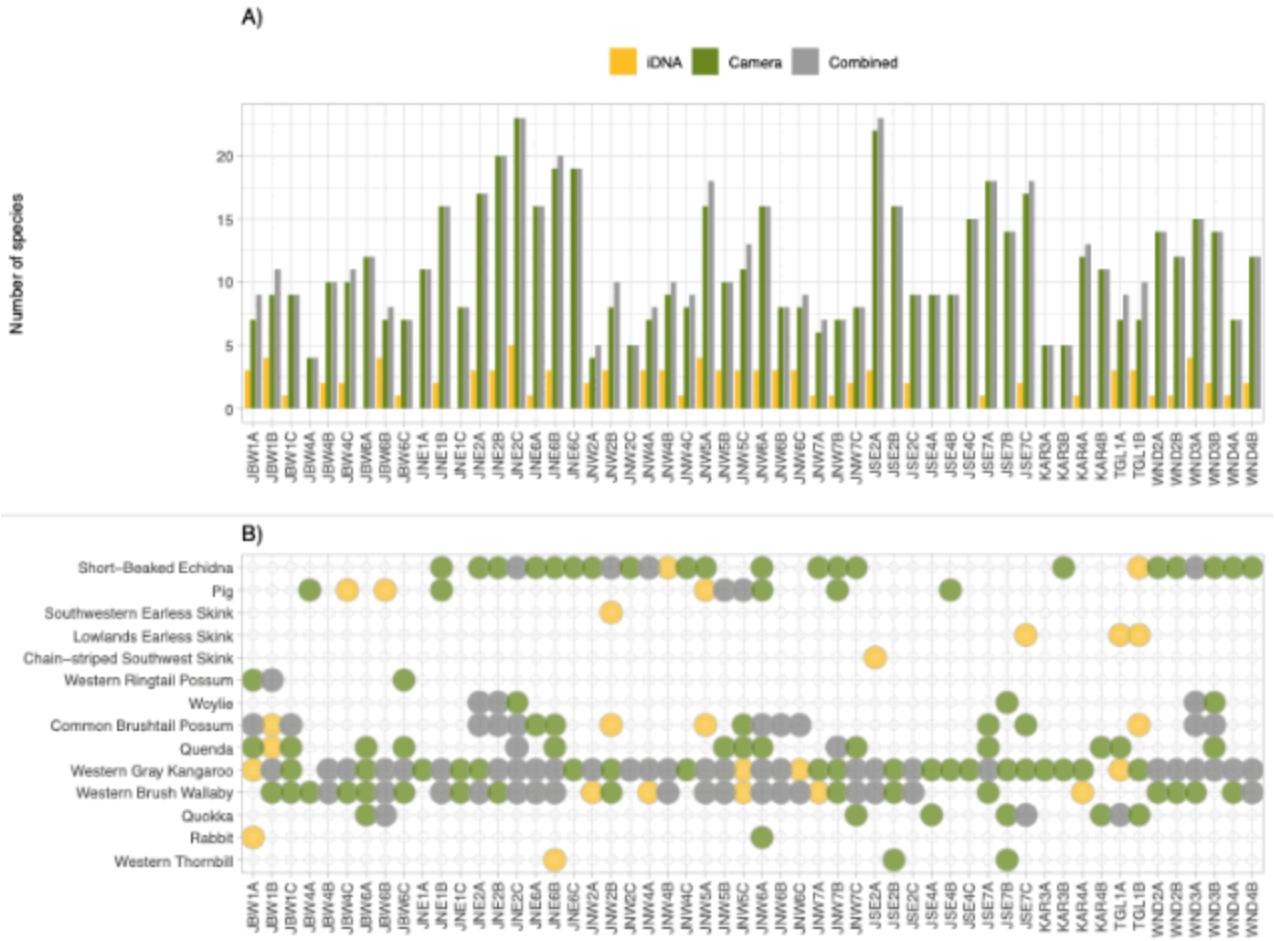


Figure 4

Comparison of forest-dwelling vertebrate species detected using iDNA and camera traps. A) Number of forest-dwelling species detected by each method across sites (including all camera detections) and B) 14 species detected via iDNA and their detection across sites. Colours represent the different methods used; yellow (iDNA), green (camera traps) and grey (detections shared across both). Site codes refer to forest ecosystem types and are as follows: JBW (Jarrah Blackwood), JNE (Jarrah North East), JNW (Jarrah North West), JSE (Jarrah South East), KAR (Karri), TGL (Tingle), WND (Wandoo). All species detected by cameras can be found in Table S4

Supplementary Files

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- SupplementaryInformationTableS1S5.xlsx