CONFIDENTIAL REPORT

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Vertebrate Report

The Great Australian Platypus Search – Victoria

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Abbreviations

Abbreviations	Description
GAPS	Great Australian Platypus Search
eDNA	environmental DNA
ALA	Atlas of Living Australia
VBA	Victorian Biodiversity Atlas
OTU	Operational taxonomic unit
ASV	Amplicon sequence variant
GRTS	Generalised Random Tessellation Stratified

Contents

Summary

- The Great Australian Platypus Search undertook eDNA sampling through citizen scientists at ~1850 sites throughout all Victorian River Basins and investigated platypus presence using qPCR. The results have been reported previously (Griffith et al. 2022; https://www.thegreataustralianplatypussearch.org). The samples have now also been assessed using the eDNA metabarcoding method to identify the presence of other vertebrate taxa.

- Samples from a total of 1850 unique sites were processed using a vertebrate metabarcoding assay, with 13,643 site detections of vertebrate taxa at the species or genus levels. A total of 6,374 site detections were aquatic taxa, the primary target taxa for these surveys.

- A total of 224 taxa were detected from the eDNA samples; 75 aquatic (mostly freshwater) taxa and 149 semi-aquatic / terrestrial taxa. In these taxa detections, 30 threatened species were identified, and there are likely to be numerous detections of other threatened species, but are only identifiable at the genus level due to low resolution in the vertebrate amplicon or lack of appropriate reference sequences.

- We compared the eDNA detection data on freshwater aquatic species with that found on the Victorian Biodiversity Atlas (VBA) for the last 20 years, comparing detections in the 29 river basins of Victoria. The results were largely comparable to the VBA data at the basin scale, and we discuss these in more detail for 44 freshwater fish species.

- New records of taxa in basins were detected from the eDNA samples for 26 native aquatic species and nine invasive species, including some threatened species (e.g. *Neochanna cleaveri*, *Tandanus tandanus*, *Maccullochella peelii*, *Gobiomorphus coxii*). However, there are also likely to be false positive site (and sample) detections and we discuss these, along with false negatives, in more detail.

- This citizen science eDNA project is spatially one of the largest eDNA surveys ever undertaken, and one of the largest vertebrate biodiversity surveys over a single season. The data provides a baseline for aquatic biodiversity that can be built upon and enable more efficient and timely future monitoring. Many lessons have also been learnt around undertaking such an ambitious eDNA program, and how future programs could be improved.

Background

Understanding spatial changes in biodiversity patterns is important for informing conservation efforts, but landscape-level data on species distributions can be logistically and financially difficult to collect. In Australia, our understanding of landscape level distributions of species has often relied upon traditional survey techniques (e.g., live-trapping, electrofishing, camera-traps, visual surveys etc) undertaken over decades, as evidenced by the various databases including the Atlas of Living Australia and the Victorian Biodiversity Atlas. However, with biodiversity declining at unprecedented rates across many of our ecosystems due to factors including anthropogenic pressures (including climate change and invasive species) and environmental catastrophes (bushfires, drought, floods), there is a growing need to undertake rapid biodiversity surveys to help inform management responses.

Environmental DNA (eDNA) is a revolutionary technology for detecting species presence within environmental samples, and shows promise as a method that can be used to rapidly undertake biodiversity surveys over large spatial scales. Environmental DNA methods enable the detection of genetic material secreted by a species into its surrounding environment. These eDNA methods have been shown repeatedly to be more sensitive and cost-effective than traditional survey methods for species detection. They have proved particularly efficient in freshwater ecosystems for the detection of amphibians (Goldberg et al. 2011), arthropods, mammals (Lugg et al. 2018), and native and invasive fish (Thomsen et al. 2012; McColl-Gausden et al. 2021). Importantly, they do not require a high level of expertise to implement the sampling methods, are non-invasive, and generally are relatively safer for humans to implement than many traditional survey methods. Environmental DNA sampling can therefore be integrated into citizen science programs to increase their cost-efficiency (Biggs et al. 2015).

A national monitoring program utilizing eDNA sampling by citizen scientists to generate landscape scale data across a range of freshwater ecosystems has been initiated by the Odonata Foundation and partner organisations. The program began in Victoria in spring and early summer of 2021 as the Great Australian Platypus Search (GAPS). Through this program, citizen scientists undertook eDNA sampling across ~2000 sites throughout the Victorian river network. The platypus was chosen as a flagship species in recognition of growing concerns of its conservation status, lack of systematic data, and capacity to engage the broader community. However, the sampling undertaken also provided the opportunity to generate landscape scale distribution data on a range of other freshwater vertebrate species, using the eDNA metabarcoding method. The results for platypuses have been communicated previously (https://www.thegreataustralianplatypussearch.org; Griffiths et al. (2022)). Here we outline results for fish, amphibian, bird, reptile and mammal species from the samples collected as part of the GAPS project.

Methods

Sampling

Environmental DNA sampling was undertaken at nearly 2000 sites throughout Victoria (Figure 1). Sites were selected as in Shackleton et al. (2021). A full description of the sampling methods is described in Griffiths et al. (2022). Briefly, water sampling was primarily undertaken by citizen scientists from September 2021 to January 2022. At each site, water samples were collected in duplicate by passing water through two 1.2 μM syringe disc filters. Sample volumes varied widely from 7 to 3430 mL (average 347 mL). Filtration was undertaken on site and a preservative (0.5 mL 10X Tris-EDTA) was added to the filters after filtering to minimize DNA degradation. Filters were then sent back to EnviroDNA for analyses, which included DNA extraction and then qPCR to detect platypus DNA (see Griffith et al. 2022) and metabarcoding using the Vertebrate assay (Riaz et al. 2011) to detect fish, amphibians, birds, reptiles and mammals.

DNA extraction and metabarcoding

DNA was extracted from the filters using a commercially available DNA extraction kit (Qiagen Power Soil Pro Kit) that minimises compounds that can inhibit the PCR reaction in environmental samples. Amplicon metabarcoding was carried out on the extracted DNA using a two-step PCR library construction method (see McColl-Gausden et al. 2020) whereby the first round of PCR employed gene-specific primers to amplify the target region and the second round incorporated sequencing adapters and unique barcodes for each sample-amplicon combination included in the library.

Initially we ran a general vertebrate assay targeting a mitochondrial 12S barcode which amplifies fish, birds, mammals, frogs and reptiles (Riaz et al. 2011). Later, a more targeted fish assay was also run for a subset of samples to improve coverage where vertebrate analysis failed or was dominated by human and agricultural taxa. Importantly, the fish assay utilized an additional layer of indexing during library construction (unique dual indexes, or UDIs) which greatly reduces the effects of sample contamination during sequencing (index-hopping/switching) and also any laboratory cross-contamination after the first PCR. Negative and positive control samples were included during library construction for each assay, including a dilution series of mock communities for the fish assay that provides an internal measurement of detection sensitivity and linearity. Sequencing was carried out primarily on a NextSeq 2000 platform (Hudson Institute of Medical Research, Clayton, VIC), with some optimisation runs carried out in-house on an Illumina iSeq 100 platform. We aimed for a sequencing depth per sample of ~80,000 reads; this is a very high depth of sequencing per sample, but was chosen to potentially increase the number of taxa detections. Unfortunately, due to the variance in vertebrate DNA concentration per sample, the average number of reads varied greatly between samples.

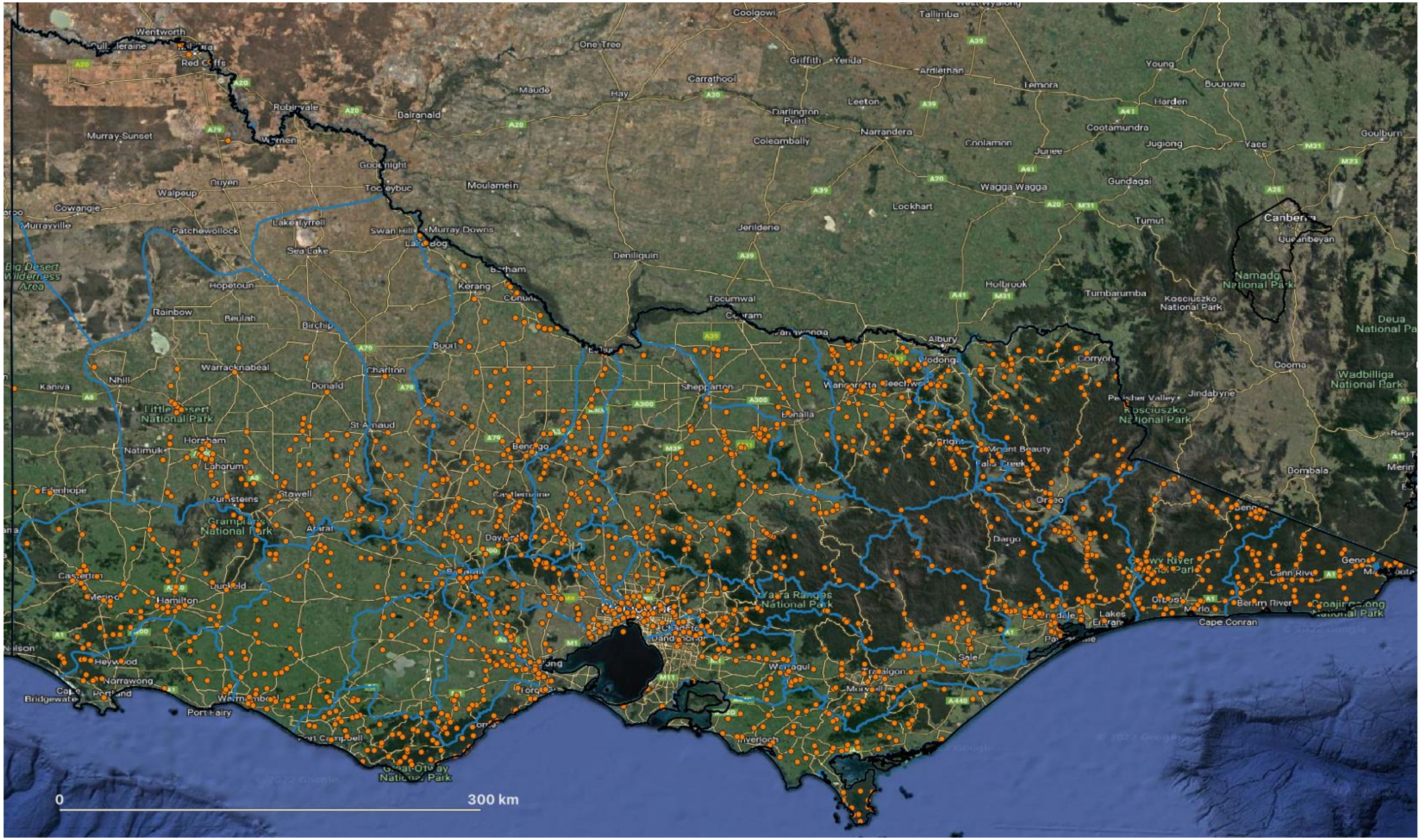

Figure 1. Location of sites surveyed (orange dots) across Victoria for the Great Australian Platypus Search Sept-Dec 2021.

Bioinformatics, data filtering and analyses

Amplicon pools were demultiplexed based on the unique barcodes which identified individual samples, and overlapping forward and reverse (R1 and R2) reads from the paired-end sequencing were merged, followed by quality control filtering to remove primer sequences, truncated reads and low-frequency sequences. DNA sequences were denoised to mitigate PCR error into unique amplicon sequence variants (ASVs), and taxonomic assignment was performed with VSEARCH's SINTAX algorithm (Edgar, 2016) whereby each Amplicon Sequence Variant (ASV) was given a taxonomic assignment using our Australian vertebrate and fish reference sequence databases, supplemented by BLAST searches. The vertebrate assay and fish assay samples were processed independently, and then combined after applying the steps below, with results presented incorporating detections from both assays (where a sample was screened with both assays).

Exclusion of samples

We excluded any sample that had under 1000 reads in total, as the number of reads distributed across each taxon in the sample would be difficult to distinguish from background levels of noise. For each plate of samples, we used controls and external data from the Victorian Biodiversity Atlas (VBA) and the Atlas of Living Australia (ALA) to assess data quality (i.e., contamination or sample misassignment). We ranked plates based on the proportion of samples with unexpected detections (e.g., for VBA/ALA observations, any detections lacking support in a given basin), and excluded 4 plates, which were clear outliers in this metric (>5% unexpected detections).

We also excluded individual samples where multiple detections were incongruent with known species distributions and there was doubt over the accuracy of the sample metadata, particularly around location coordinates (8 for the vertebrate assay, 81 for the fish assay). A third category of samples that were excluded were those where the vertebrate assay resulted in very low read counts (dropouts), but the fish assay resulted in uncommonly high species richness, with several detections incongruent with known species distributions. It is unclear what the cause of this phenomenon was, but we believe that these samples are likely to be dropouts and that the reads were molecular artifacts as a consequence of the sample having insufficient levels of amplifiable DNA.

Taxon-specific filtering

For the vertebrate amplicon analysis, where library preparation at the time did not include unique dual indexes (UDIs), we observed strong bimodality in read count distributions for common taxa consistent with low-level index switching. To mitigate this, we applied taxon-specific filters (which ranged between 0 - 0.2% of reads across samples for a taxon) based on mixture modelling of read count distributions, which strongly reduced the frequency of unexpected (out of known basin) detections. For the fish amplicon, where UDIs were used in library preparation, no filtering thresholds were applied.

(Note: In hindsight, the benefits of including unique dual indexes (which adds significant upfront costs) outweighs the cost as the scale of index-hopping becomes more apparent with sequencing depth and increases the complexity of distinguishing signal from noise.)

Manual taxonomic assignments

For some aquatic fish taxa that are closely related, we further investigated ASV assignment by viewing individual ASVs at the genus level, their phylogenetic relationship (using neighbour-joining distance method) with species reference sequences from the genus, and the known species distributions. This analysis was undertaken for several native species groups including little / dwarf galaxias (*Galaxiella pusilla*, *G. toourtkoourt*), estuary perch / Australian bass (*Macquaria colonorum*, *M. novemaculeata*), river blackfish / two spined blackfish (*Gadopsis marmoratus*, *G. bispinosus*), southern / variegated / Yarra pygmy perch (*Nannoperca australis*, *N. variegata*, *N. obscura*) and the various *Galaxias* species. Some ASVs could not be assigned to species and therefore were left at the genus level (e.g. *Gadopsis* sp., *Nannoperca* sp.) even though other ASVs could be assigned to species. Due to low differentiation between ASVs within the Mountain Galaxias species complex, all detections were assigned as "*G. olidus* species complex". For *Hypseleotris*, all assignments were left at the genus level due to the known hybridisation within this group and low differentiation between ASVs.

We excluded assignments that were stuck at taxonomic ranks above genus as they were not informative, with the exception of Anatidae (ducks, geese and swans) as this family contains many widely-occurring species which may be of interest despite the poor resolution.

False Positive Detections in eDNA surveys

When we discuss false positives in eDNA detections, we first need to differentiate between false positive samples (e.g., errant detection in an individual sample) and false positive sites (e.g., errant detection at an unoccupied site). eDNA methods, such as eDNA metabarcoding, test for DNA presence in a sample, and it is possible to have a true-positive sample (e.g., the detection of a species DNA) when the species itself is not present at a site. Technically, this is not a false positive eDNA detection, as the eDNA method did exactly what it was intended to do – detect the DNA of a species within a sample. This is an important point to make, as DNA of a species could occur through eDNA transport in waterways (upstream to downstream) or through other species movement patterns (e.g. humans or other wildlife). Darling et al. (2021) provides an excellent review of false positives in eDNA surveys and what they actually mean.

We also highlight that in citizen science programs, such as that undertaken in this project, the general public are taking samples at predetermined sites. If the sample is not taken at the allocated site or there are sample mix-ups in the laboratory (due to poorly collated metadata), then this can result in a false positive site, but not necessarily a false positive sample - again, this is not the fault of the eDNA method, but rather human error.

In line with the above, for this project, we identify two different types of false positives. The first is related to laboratory processes (contamination, inadequate reference sequences, low resolution of the amplicon sequence(s), index switching) that results in a false positive sample, while the second is a false positive site (species detection when the site is unoccupied by the species). We have attempted to deal with the first as best we can using a variety of laboratory methods as mentioned above (negative controls, mock communities (for the fish assay), filtering to reduce the effects of index switching etc). However, low resolution of ASVs and an incomplete reference library (describing both inter and intraspecific variation) for the large spatial scale of this survey presents extra challenges. We try to differentiate between the two types of false positives when discussing the results for key aquatic species below, although it can be difficult to differentiate.

VBA / ALA comparisons

We compared the eDNA detections with the Victorian Biodiversity Atlas (https://vba.biodiversity.vic.gov.au), and for terrestrial / semi-aquatic taxa, with the Atlas of Living Australia (www.ala.org.au). These comparisons were undertaken to understand how well the eDNA data matched previous records within these databases (and to highlight potential new records). We restricted the comparisons with the VBA for aquatic species to the last 20 years (2002-2021) from when the eDNA surveys were undertaken. We mostly only compare freshwater aquatic species, as marine and estuarine species were not specifically targeted in the sampling design (although we do report on some coastal species here).

The primary focus of this report is freshwater species, given the sampling design was specifically developed to target these taxa. However, data was also collected on a wide-range of terrestrial, semi-aquatic and estuarine species and we present an overview of these detections. These detections can be further investigated in the R Shiny app that accompanies this report (https://envirodna.shinyapps.io/GVWS_release/).

Findings

After quality control filtering and removing detections that were stuck at higher taxonomic levels, we obtained sequence reads from 1,850 sites across Victoria, with 1,813 sites producing >1,000 sequence reads, and 1,771 producing >5,000 sequence reads. From the eDNA samples, there were 13,643 detections of vertebrate taxa at genus or species levels, with 6,374 detections of aquatic taxa (classes Chondrichthyes, Hyperoartia, and Actinopteri). Across all samples, 75 aquatic taxa were detected, in addition to 149 terrestrial / semi-aquatic (non-target) taxa. The vast majority (97%) of detected aquatic species belonged to the class Actinopteri (ray-finned fish). Detections of terrestrial taxa were more evenly distributed among taxonomic classes, with birds being the most frequently detected taxon (taxa detected in Amphibia = 12%; Aves = 50%; Mammalia = 30%; Reptilia = 9%). However, this is perhaps unsurprising, given avian diversity relative to that of other groups. Indeed, when focusing on the total number of detections per class, mammals were the most frequently detected group (detections per class: Amphibia = 13%; Aves = 39%; Mammalia = 47%; Reptilia = 1%).

Among aquatic taxa detected via eDNA sampling, 13 (18%) species were not native to Australia. A comparable proportion (17%; n = 23) of terrestrial species were non-native. eDNA sampling also detected species listed as threatened in Victoria (FFG Act) and nationally (EPBC Act). Among aquatic species, 11 were federally listed, with 18 listed in Victoria. Nineteen terrestrial species were federally listed, whereas 12 were listed at the state level. There were detections of taxa at the genus level that likely also represent threatened species at the state or federal level e.g. for aquatic taxa, *Gadopsis marmoratus* (upper Wannon), *Nannoperca* sp. 1 (Flinders pygmy perch), *Hypseleotris compressa* (Empire gudgeon), and the various Mountain galaxias species found in the coastal basin side of Gippsland. Unfortunately, the assay(s) could not differentiate between these species due to their close phylogenetic relationships with other sister species. Nonetheless, these detections provide information that may be useful for helping delineate their distributions.

A complete list of species detected can be found in Appendix 1 (with number of detections at the site level). All the site detections for taxa can also be found at the following location in an R Shiny app (https://envirodna.shinyapps.io/GVWS_release/).

Comparison between eDNA sampling and existing databases

The table below (Table 1) compares the number of Victorian river basins (29 in total) in which each taxa was detected with eDNA sampling, compared to the same metric derived from Victorian Biodiversity Atlas data. Species-specific differences and geographic overlap between the two data sources are elaborated upon further in species accounts below, which have been prepared for 44 aquatic taxa. Overall, however, there appears to be generally good correspondence between data sources, although there are potential false positives for some species which we highlight and need further investigation.

Table 1. Number of basins in which aquatic species (Chondrichthyes, Hyperoartia, and Actinopteri) were detected via eDNA sampling, compared to detections in the Victorian Biodiversity Atlas (VBA) recorded after 2003. Columns show, from left to right, the number of basins in which both sampling methods detected a species; only eDNA sampling detected the species; neither method detected the species; and only VBA records detected the species. The final two columns show the total number of basins in which a species was detected in each dataset.

Status	Class	Species	eDNA & VBA	eDNA	Neither	VBA	Occupied basins	
							eDNA total	VBA total
Introduced	Actinopterygii	*Acanthogobius flavimanus*	1	0	24	4	1	5
		Carassius auratus	23	1	3	2	24	25

Status	Class	Species	eDNA & VBA	eDNA	Neither	VBA	Occupied basins	
							eDNA total	VBA total
		Cyprinus carpio	24	1	4	0	25	24
		Gambusia holbrooki	24	1	1	3	25	27
		Misgurnus anguillicaudatus	9	3	15	2	12	11
		Oncorhynchus mykiss	12	5	7	5	17	17
		Perca fluviatilis	24	2	1	2	26	26
		Rutilus rutilus	7	4	16	2	11	9
		Salmo trutta	24	2	2	1	26	25
		Tinca tinca	15	2	7	5	17	20
Native		*Acanthopagrus butcheri*	7	1	12	9	8	16
		Afurcagobius tamarensis	6	1	19	3	7	9
		Aldrichetta forsteri	4	0	16	9	4	13
		Anguilla australis	22	4	3	0	26	22
		Anguilla reinhardtii	7	5	16	1	12	8
		Arenigobius bifrenatus	3	1	18	7	4	10
		Arenigobius frenatus	1	3	25	0	4	1
		Arripis trutta	1	1	25	2	2	3
		Atherinosoma microstoma	4	1	21	3	5	7
		Bidyanus bidyanus	1	0	22	6	1	7
		Gadopsis bispinosus	5	0	23	1	5	6
		Gadopsis marmoratus	21	1	2	5	22	26
		Galaxias brevipinnis	15	6	6	2	21	17
		Galaxias maculatus	22	2	5	0	24	22
		Galaxias rostratus	1	0	27	1	1	2
		Galaxias truttaceus	8	0	13	8	8	16
		Galaxiella pusilla	5	0	21	3	5	8
		Galaxiella toourtkoourt	4	0	25	0	4	4
		Gobiomorphus coxii	1	0	28	0	1	1
		Gobiopterus semivestitus	2	1	21	5	3	7
		Hypseleotris spp.	11	4	12	2	15	13
		Maccullochella macquariensis	2	0	24	3	2	5
		Maccullochella peelii	8	1	17	3	9	11
		Macquaria ambigua	8	3	14	4	11	12
		Macquaria australasica	4	0	16	9	4	13
		Macquaria colonorum	5	4	15	5	9	10
		Melanotaenia fluviatilis	4	0	23	2	4	6
		Mugil cephalus	2	3	17	7	5	9
		Mugilogobius platynotus	1	0	27	1	1	2
		Nannoperca australis	18	9	1	1	27	19
		Nannoperca variegata	1	0	28	0	1	1

Status	Class	Species	eDNA & VBA	eDNA	Neither	VBA	Occupied basins	
							eDNA total	VBA total
		Nematalosa erebi	3	1	24	1	4	4
		Neochanna cleaveri	0	1	26	2	1	2
		Philypnodon grandiceps	26	1	0	2	27	28
		Philypnodon macrostomus	5	4	19	1	9	6
		Prototroctes maraena	3	0	17	9	3	12
		Pseudaphritis urvillii	15	2	9	3	17	18
		Pseudogobius olorum	1	9	19	0	10	1
		Retropinna semoni	26	0	1	2	26	28
		Tandanus tandanus	3	3	19	4	6	7
		Tasmanogobius lasti	1	2	25	1	3	2
	Hyperoartia	*Geotria australis*	5	3	18	3	8	8

An alternative method to compare basin-level detections between eDNA sampling and historic data in the VBA (for the last 20 years) is to plot the number of species detected in each basin by each sampling method. Doing so for the 52 aquatic taxa present in both datasets (those in Table 1) reveals a strong positive relationship between the number of taxa detected in each basin. This highlights the potential of eDNA; a single survey undertaken over such a broad spatial scale was able to capture a large proportion of over 20 years of VBA detections.

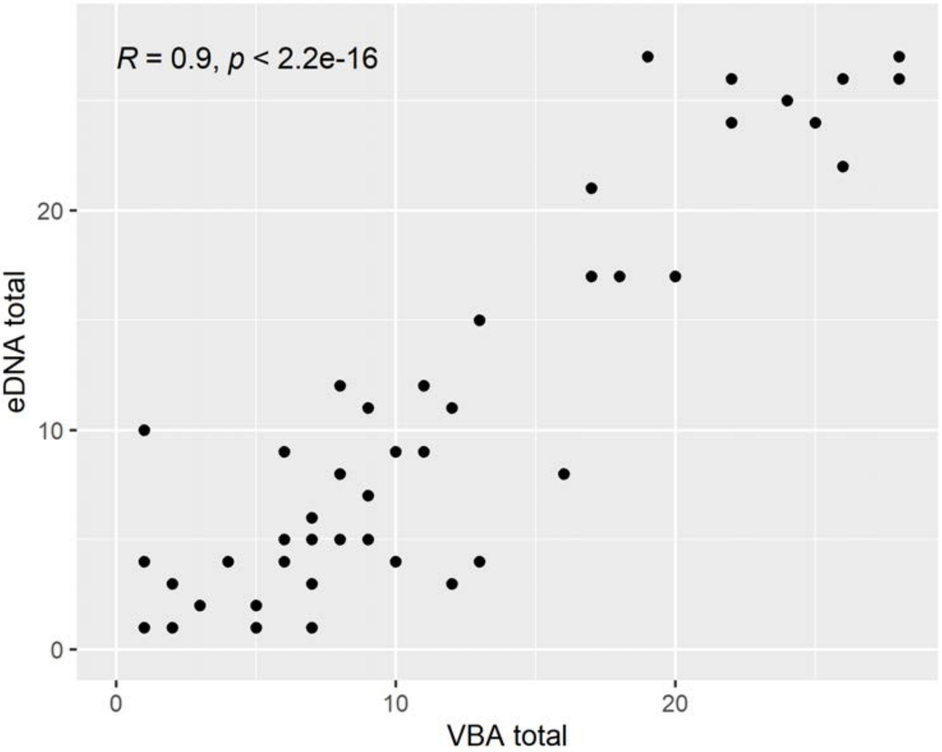

Figure 2. Number of basins occupied by 50 freshwater taxa (Hyperoartia and Actinopteri) based on eDNA sampling and records from the Victorian Biodiversity Atlas (post-2003).

Species Accounts

Below we briefly summarise eDNA detections in the context of records within the Victorian Biodiversity Atlas. Each species map shows detections from both data sources, overlaid on 29 Victorian River Basins. The color of each basin corresponds to the proportion of eDNA samples in which a species was detected. We undertake this for 44 aquatic (fish) taxa, and also include several detection maps for terrestrial or semi-aquatic threatened species detected from the eDNA samples.

Large-bodied native species

Short-finned eel, *Anguilla australis*

Anguilla australis is a widespread species, occupying 22 river basins based on the records in the VBA. This species was detected in 26 river basins with eDNA sampling, 22 of which were observed previously according to VBA. eDNA sampling therefore detected *Anguilla australis* in 4 river basins for which there were no corresponding VBA records. The only basins in which *Anguilla australis* was not detected with eDNA sampling are Avoca River, Mallee and Millicent Coast. This was one of the most common species detected in the eDNA samples. Some detections occurred in basins that are part of the Murray Darling Basin, and these should be reviewed for potential false positive site detections.

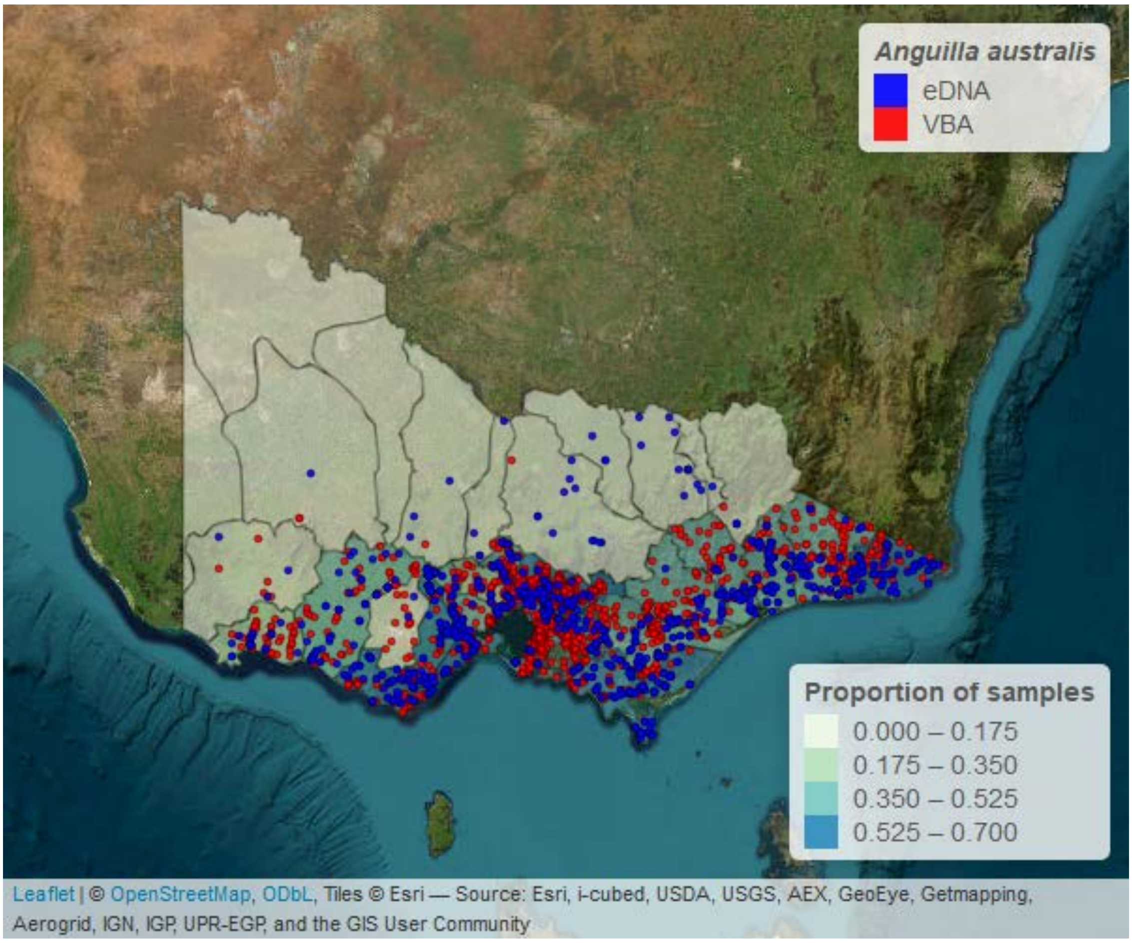

Figure 3. Detections of *A. australis* with eDNA (blue dots) and those recorded in the VBA (red dots) for the last 20 years (2003-2023). Basins are colour coded for the proportion of eDNA detections positive for the species based on the number of sites sampled within the basin.

Long-finned eel, *Anguilla reinhardtii*

Anguilla reinhardtii occupies 8 river basins based on the records in the VBA. This species was detected in 12 river basins with eDNA sampling, 7 of which were observed previously according to VBA. eDNA sampling therefore detected *Anguilla reinhardtii* in 5 river basins for which there were no corresponding VBA records. These were generally single detections in each basin and could possibly represent false positive site detections.

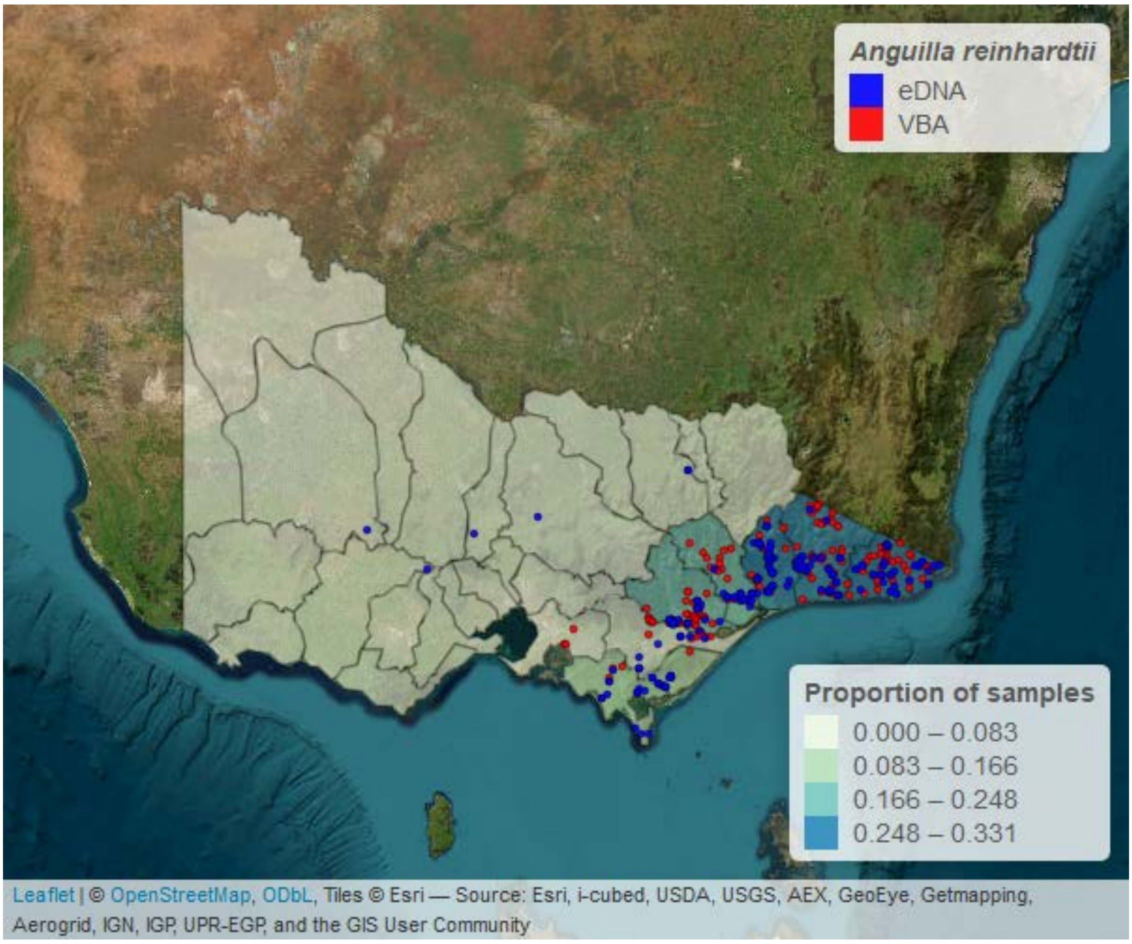

Figure 4. Detections of *A. reinhardtii* with eDNA (blue dots) and those recorded in the VBA (red dots) for the last 20 years (2003-2023). Basins are colour coded for the proportion of eDNA detections positive for the species based on the number of sites sampled within the basin.

Silver perch, *Bidyanus bidyanus* (FFG listed)

Bidyanus bidyanus occupies 7 river basins based on the records in the VBA. This species was detected in 1 river basin with eDNA sampling (Broken River), where the species has been recorded previously based on VBA records. There were very few eDNA samples collected from sites in the Murray River, and this likely reduced the detections using the eDNA method across basins, given that many previous records for this species are from the Murray River.

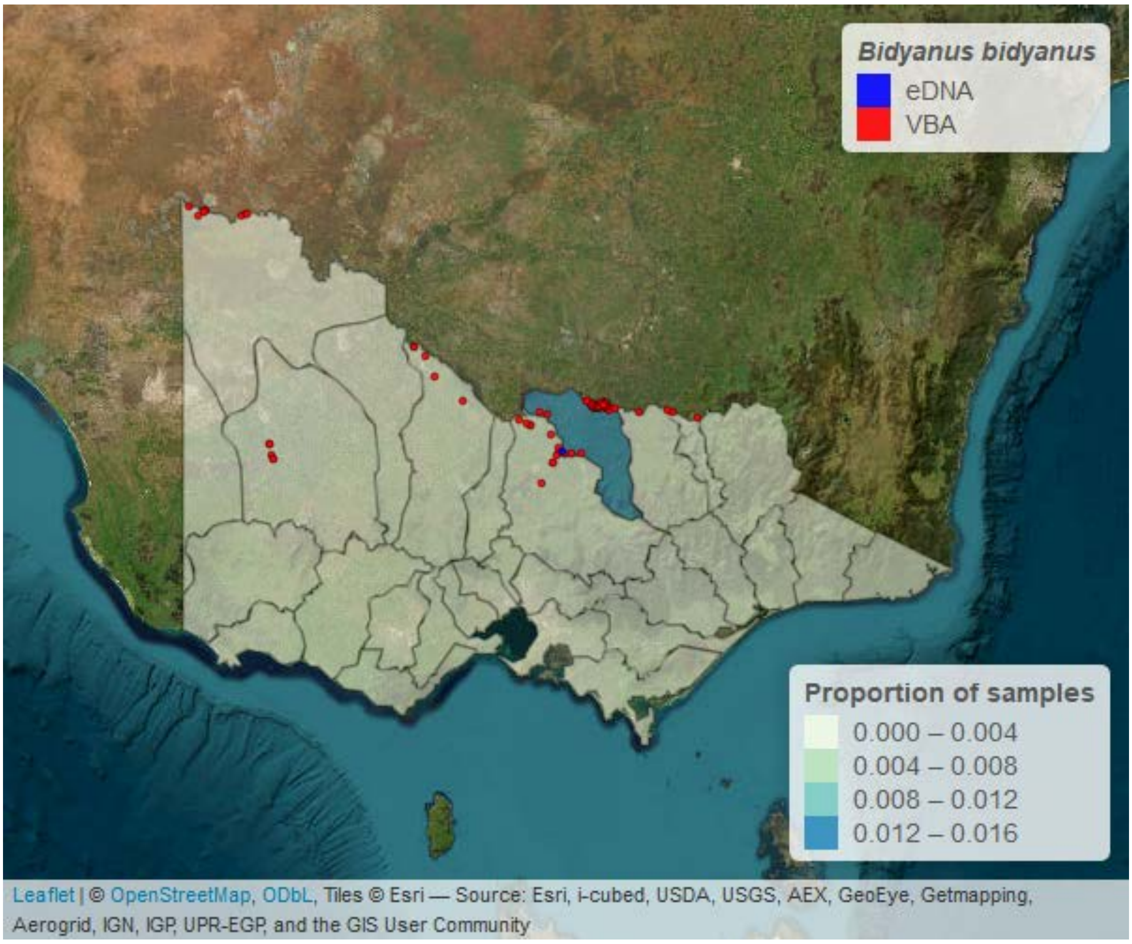

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Figure 5. Detections of *B. bidyanus* with eDNA (blue dots) and those recorded in the VBA (red dots) for the last 20 years (2003-2023). Basins are colour coded for the proportion of eDNA detections positive for the species based on the number of sites sampled within the basin.

River blackfish, *Gadopsis marmoratus*

Gadopsis marmoratus is a widespread species, occupying 26 river basins based on the records in the VBA. This species was detected in 22 river basins with eDNA sampling, 21 of which were observed previously according to VBA. eDNA sampling therefore detected *Gadopsis marmoratus* in 1 river basin (Maribyrnong River) for which there were no corresponding VBA records. There were, however, detections stuck at the genus level (green dots in Figure 6), and these could not be allocated to either *G. marmoratus* or its close sister species, *G. bispinosus*. Interestingly, *G. marmoratus* was detected in the Wannon River, and likely represents the threatened Wannon River Blackfish. Further work is needed to understand the intraspecific / interspecific variation (ASVs) within and between both *G. marmoratus* and *G. bispinosus*.

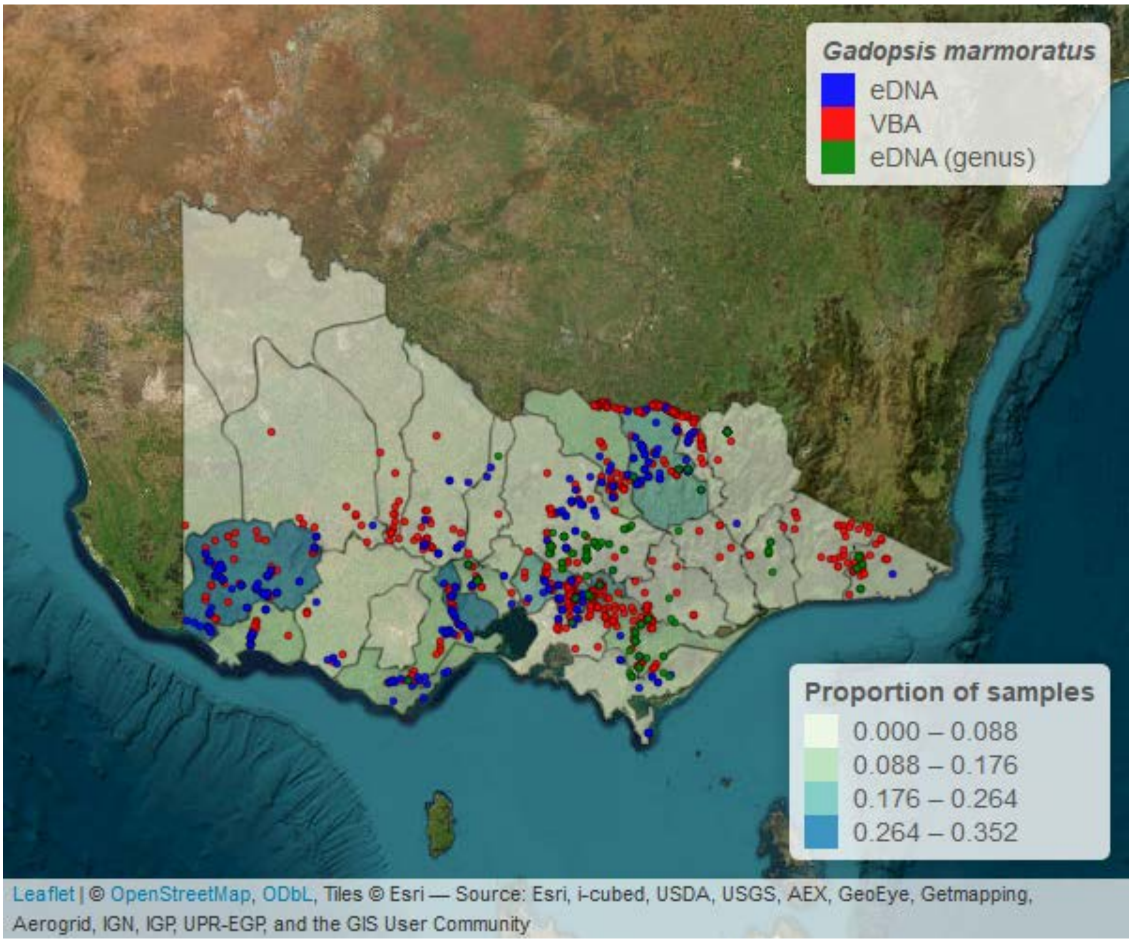

Figure 6. Detections of *G. marmoratus* with eDNA (blue dots), eDNA detections at the genus level (green dots) and those recorded in the VBA (red dots) for the last 20 years (2003-2023). Basins are colour coded for the proportion of eDNA detections positive for the species based on the number of sites sampled within the basin.

Two-spined blackfish, *Gadopsis bispinosus*

Gadopsis bispinosus occupies 6 river basins based on the records in the VBA. This species was detected in 5 river basins with eDNA sampling, all 5 of which were observed previously according to VBA. *Gadopsis bispinosus* was detected in the following basins with eDNA sampling: Broken River, Goulburn River, Kiewa River, Ovens River and Upper Murray River. Again, there were detections stuck at the genus level, which will represent detections of *G. bispinosus* and / or *G. marmoratus*. As above, further work is needed to understand the inter- and intra-specific variation (ASVs) for these two closely related species.

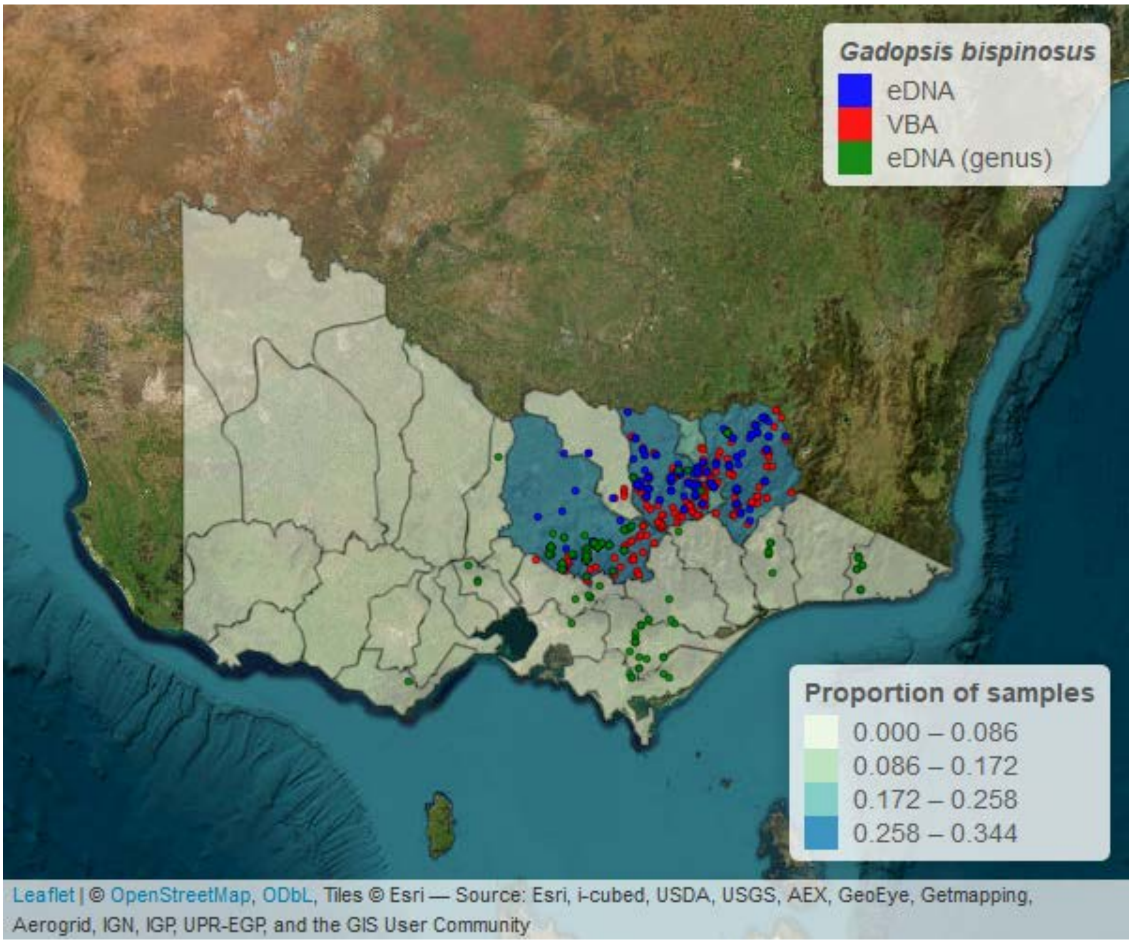

Figure 7. Detections of *G. bispinosus* with eDNA (blue dots), eDNA detections at the genus level (green dots) and those recorded in the VBA (red dots) for the last 20 years (2003-2023). Basins are colour coded for the proportion of eDNA detections positive for the species based on the number of sites sampled within the basin.

Pouched lamprey, *Geotria australis*

Geotria australis occupies 8 river basins based on the records in the VBA. This species was detected in 8 river basins with eDNA sampling, 5 of which were observed previously according to VBA. eDNA sampling therefore detected *Geotria australis* in 3 river basins for which there were no corresponding VBA records.

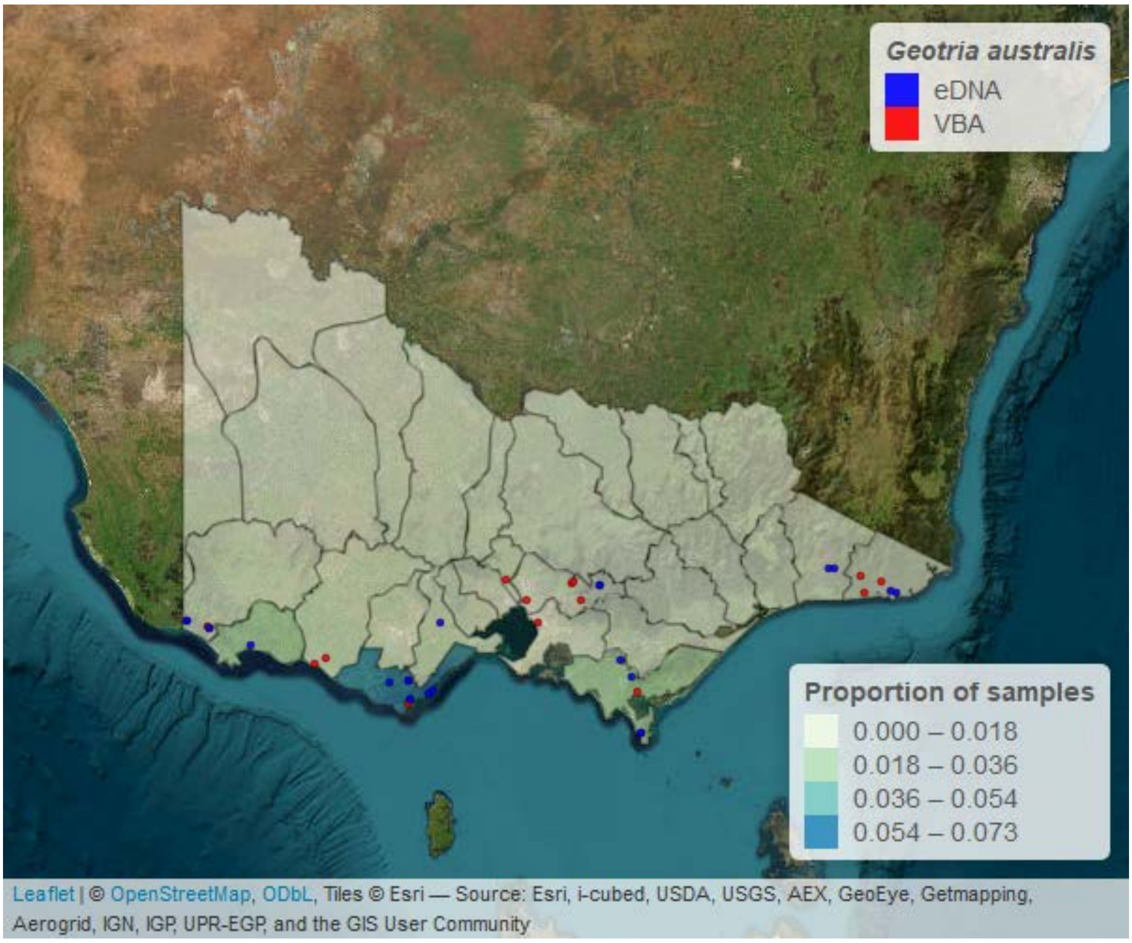

Figure 8. Detections of *G. australis* with eDNA (blue dots) and those recorded in the VBA (red dots) for the last 20 years (2003-2023). Basins are colour coded for the proportion of eDNA detections positive for the species based on the number of sites sampled within the basin.

Trout cod, *Maccullochella macquariensis* (FFG listed)

Maccullochella macquariensis occupies 5 river basins based on the records in the VBA. This species was detected in 2 river basins with eDNA sampling (Goulburn River and Ovens River), both of which have been observed previously according to VBA records. This species is known primarily from the Murray River, and there were very few sites sampled in the Murray River, which likely accounts for the lower number of basins where the species was detected.

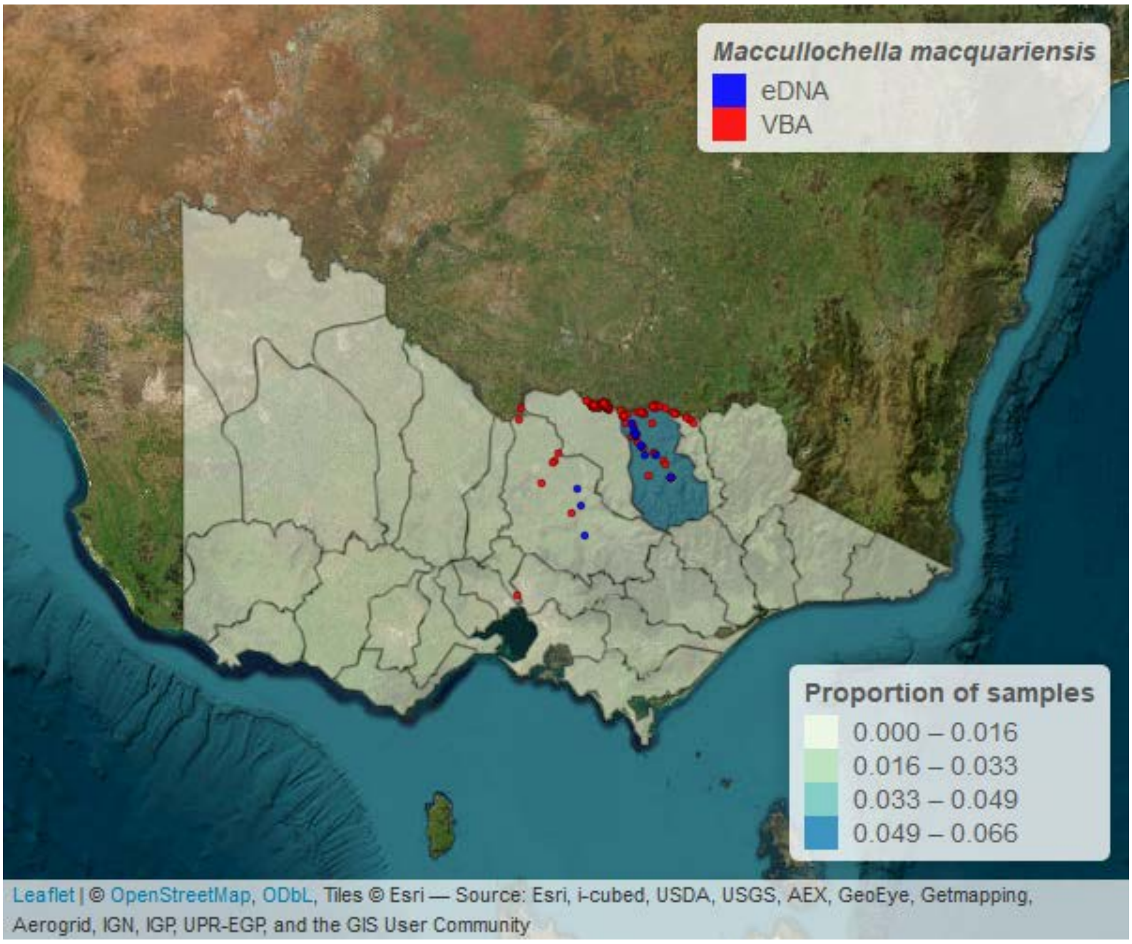

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Figure 9. Detections of *M. macquariensis* with eDNA (blue dots) and those recorded in the VBA (red dots) for the last 20 years (2003-2023). Basins are colour coded for the proportion of eDNA detections positive for the species based on the number of sites sampled within the basin.

Murray cod, *Maccullochella peelii* (FFG listed)

Maccullochella peelii occupies 11 river basins based on the records in the VBA. This species was detected in 9 river basins with eDNA sampling, 8 of which were observed previously according to VBA. eDNA sampling therefore detected *Maccullochella peelii* in 1 river basin for which there were no corresponding VBA records. This was an odd detection and may represent a false positive detection. Historically the species is found in the Murray River, and with few sites sampled in the Murray River, this likely reduced the number of basin level detections when compared with the VBA.

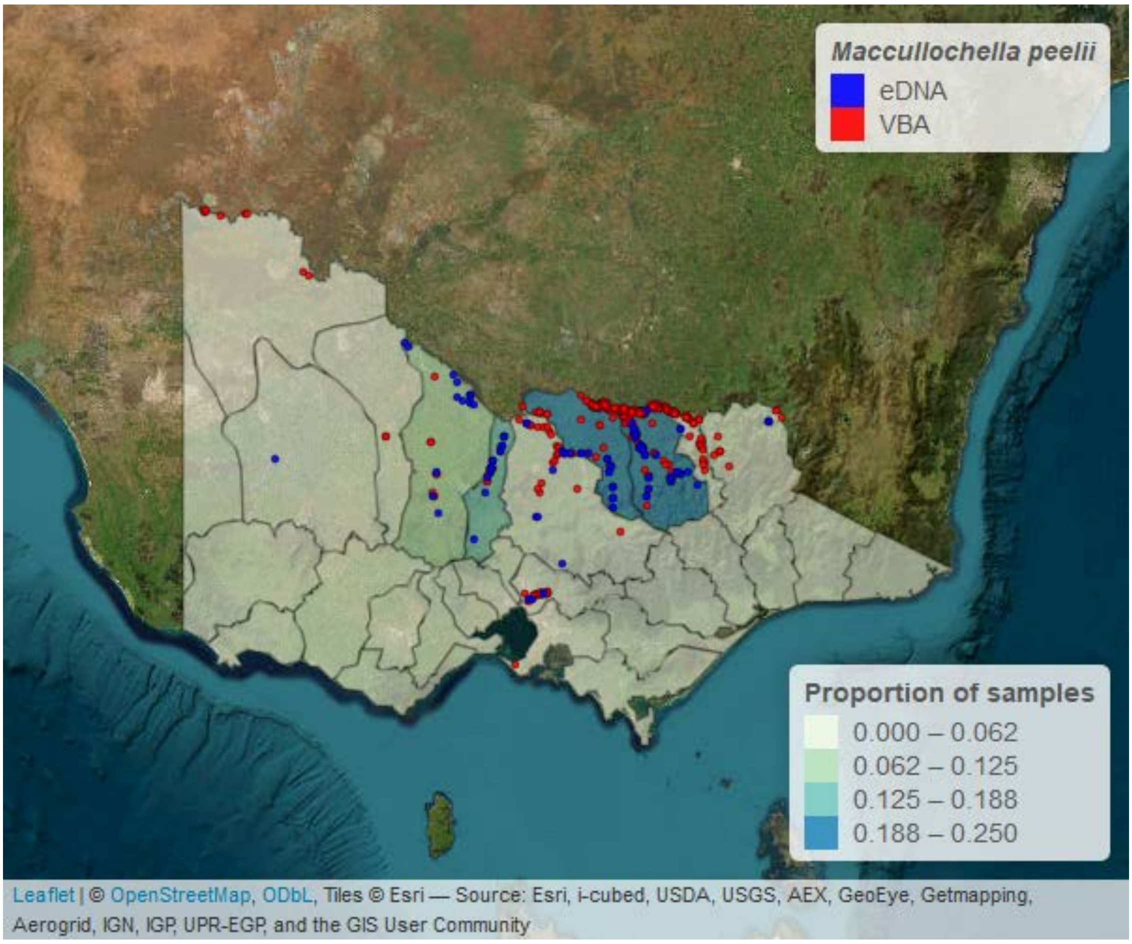

Figure 10. Detections of *M. peelii* with eDNA (blue dots) and those recorded in the VBA (red dots) for the last 20 years (2003-2023). Basins are colour coded for the proportion of eDNA detections positive for the species based on the number of sites sampled within the basin.

Golden perch, *Macquaria ambigua*

Macquaria ambigua occupies 12 river basins based on the records in the VBA. This species was detected in 11 river basins with eDNA sampling, 8 of which were observed previously according to VBA. eDNA sampling therefore detected *Macquaria ambigua* in 3 river basins for which there were no corresponding VBA records. These detections warrant further investigation, and may represent false positive site detections or individuals that have been released into these waterways by recreational fishers (golden perch are a stocked species in some Victorian waterways).

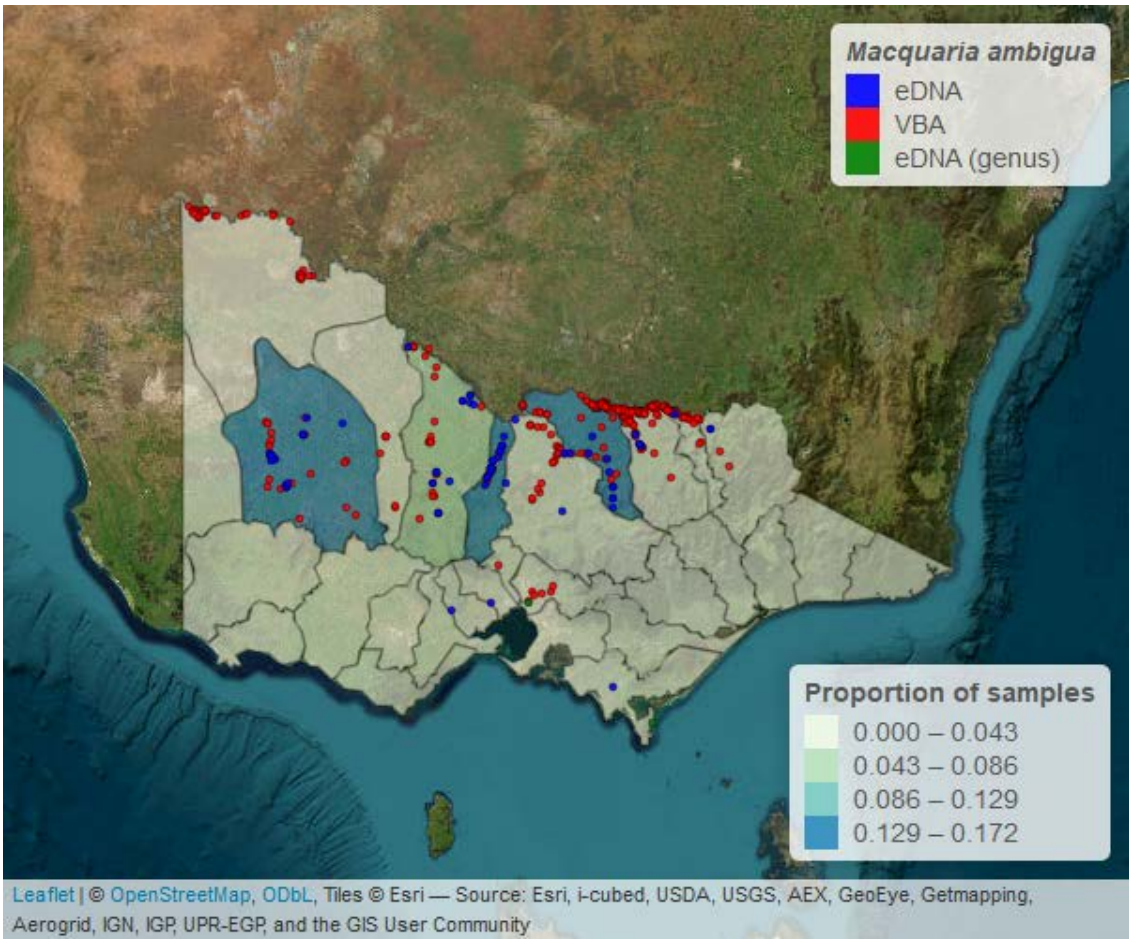

Figure 11. Detections of *M. ambigua* with eDNA (blue dots) and those recorded in the VBA (red dots) for the last 20 years (2003-2023). Basins are colour coded for the proportion of eDNA detections positive for the species based on the number of sites sampled within the basin.

Macquarie perch, *Macquaria australasica* (FFG listed)

Macquaria australasica occupies 13 river basins based on the records in the VBA. This species was detected in 4 river basins with eDNA sampling, all 4 of which were observed previously according to VBA. *Macquaria australasica* was detected in the following basins with eDNA sampling: Broken River, Goulburn River, Ovens River and Yarra River. There are more VBA records in these basins, compared with only 1 or 2 detections in the other 9 basins where there was no eDNA detections.

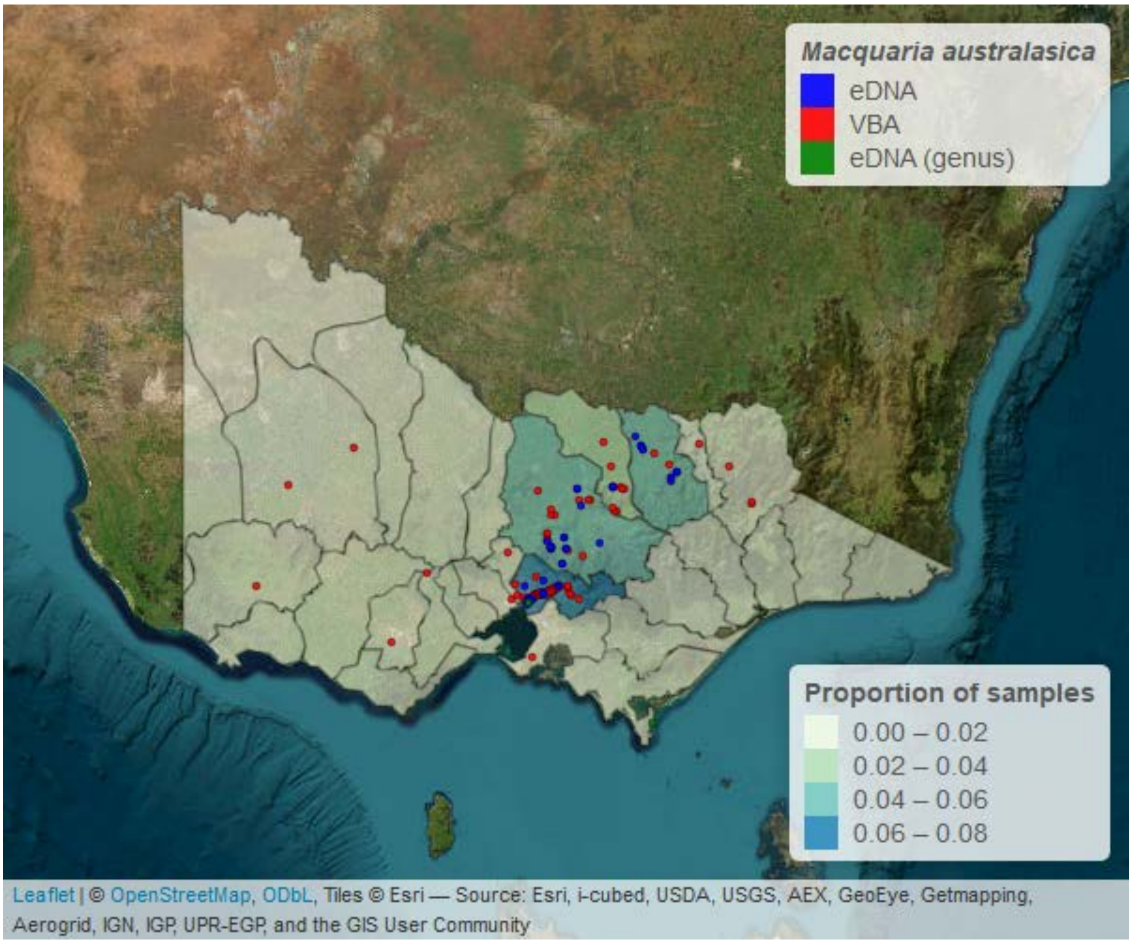

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Figure 12. Detections of *M. australasica* with eDNA (blue dots) and those recorded in the VBA (red dots) for the last 20 years (2003-2023). Basins are colour coded for the proportion of eDNA detections positive for the species based on the number of sites sampled within the basin.

Congoli (Tupong), *Pseudaphritis urvillii*

Pseudaphritis urvillii occupies 18 river basins based on the records in the VBA. This species was detected in 17 river basins with eDNA sampling, 15 of which were observed previously according to VBA. eDNA sampling therefore detected *Pseudaphritis urvillii* in 2 river basins for which there were no corresponding VBA records. These were single site detections in two basins that are part of the Murray Darling Basin, and therefore may represent false positive site detections.

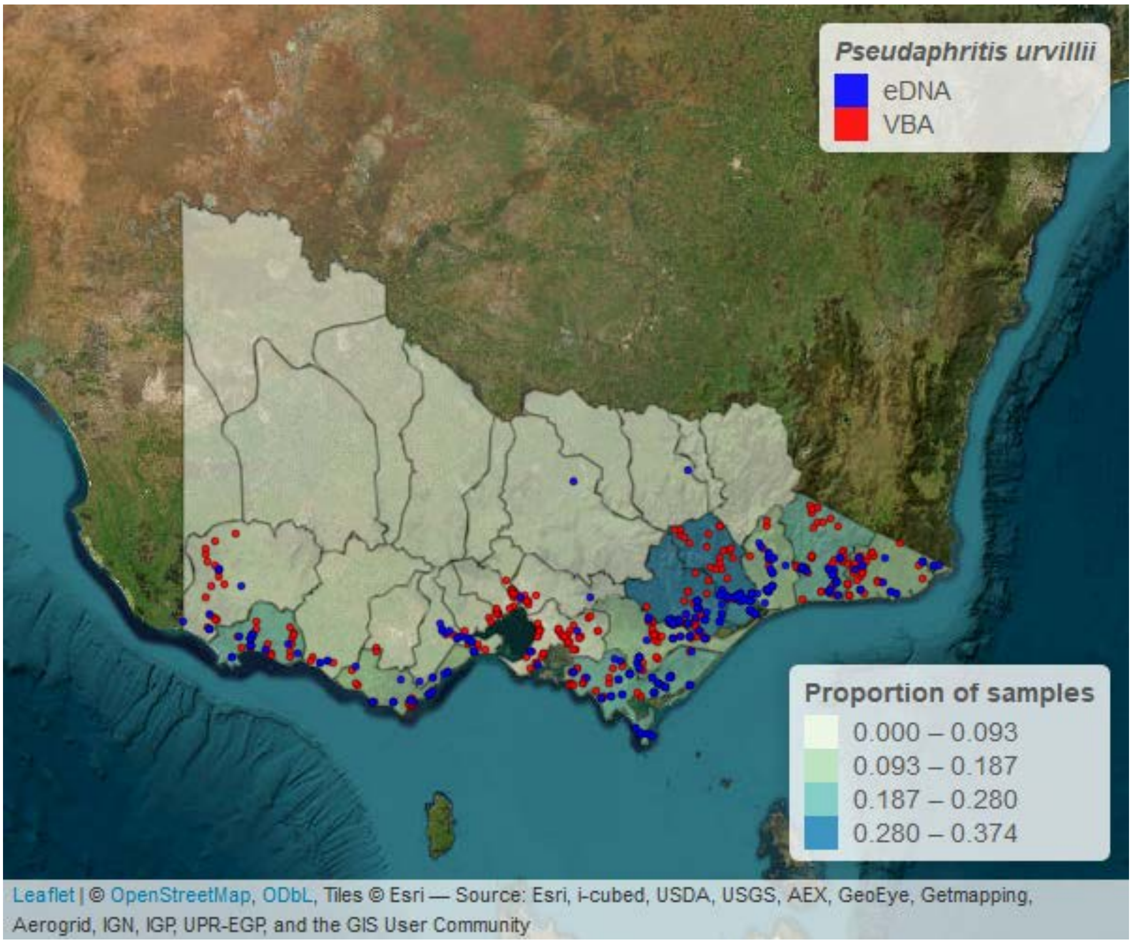

Figure 13. Detections of *P. urvillii* with eDNA (blue dots) and those recorded in the VBA (red dots) for the last 20 years (2003-2023). Basins are colour coded for the proportion of eDNA detections positive for the species based on the number of sites sampled within the basin.

Small-bodied native species

Mountain galaxias, *Galaxias olidus* species complex

The *Galaxias olidus* species complex comprises at least 15 very closely related species, with the majority found in Victoria (Raadik 2014). Unfortunately, these species cannot be separated using the Vertebrate (or fish) assay(s), due to overlapping haplotypes amongst species. We therefore provide in Figure 14 all the detections that belong to the *G. olidus* species complex (VBA records are not shown because they are classified at the species level). Some are likely to be detections of known threatened species (e.g. Barred Galaxias, East Gippsland Galaxias, Shaw Galaxias, Tapered Galaxias, Dargo Galaxias), particular those coastal basin side in Gippsland. These potentially warrant further investigation.

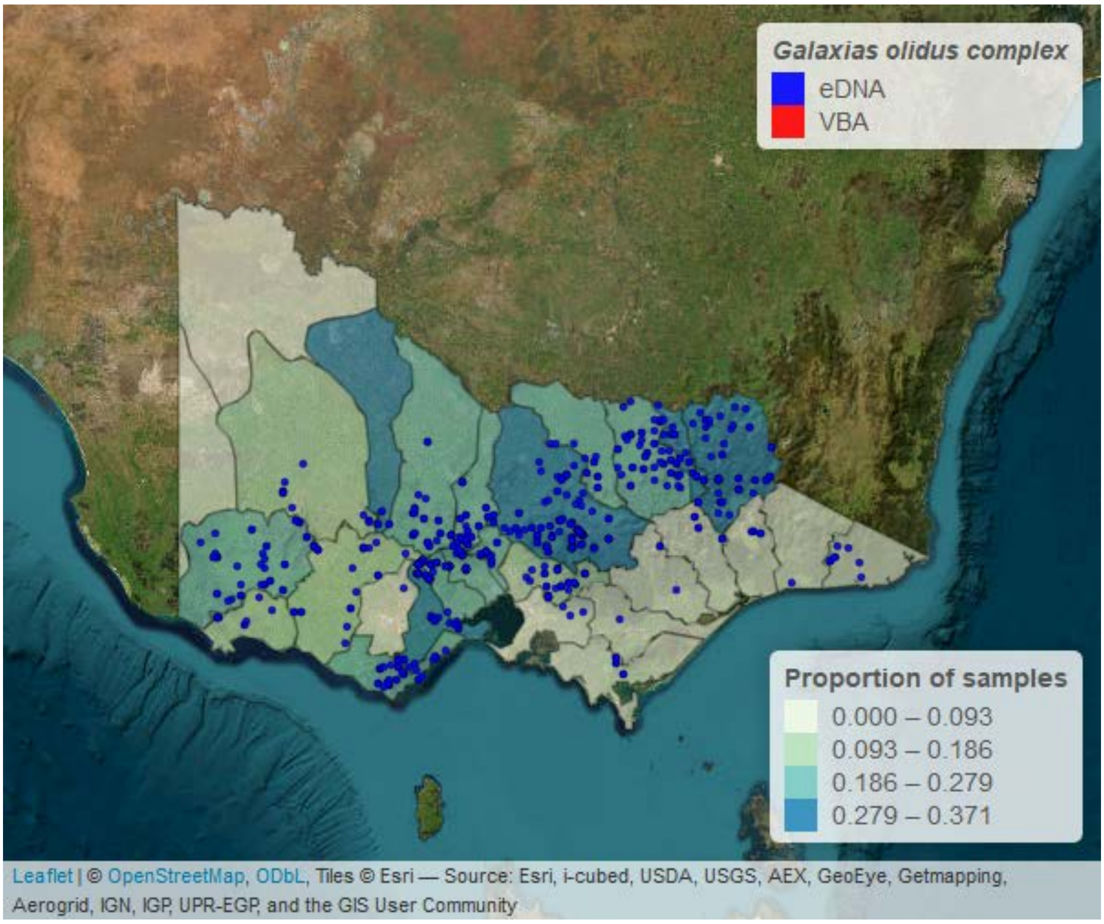

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Figure 14. Detections of *G. olidus* species complex with eDNA (blue dots). Basins are colour coded for the proportion of eDNA detections positive for the species based on the number of sites sampled within the basin. There are no corresponding VBA records shown.

Dwarf galaxias, *Galaxiella pusilla* (FFG listed)

Galaxiella pusilla occupies 8 river basins based on the records in the VBA. This species was detected in 5 river basins with eDNA sampling, all 5 of which were observed previously according to VBA. *Galaxiella pusilla* was detected in the following basins with eDNA sampling: Bunyip River, Latrobe River, Mitchell River, South Gippsland and Yarra River. The species is a wetland/drainage channel specialist, and those habitats were not targeted in this citizen science sampling program. The detection in the Yarra basin is surprising and is a possible false positive site detection.

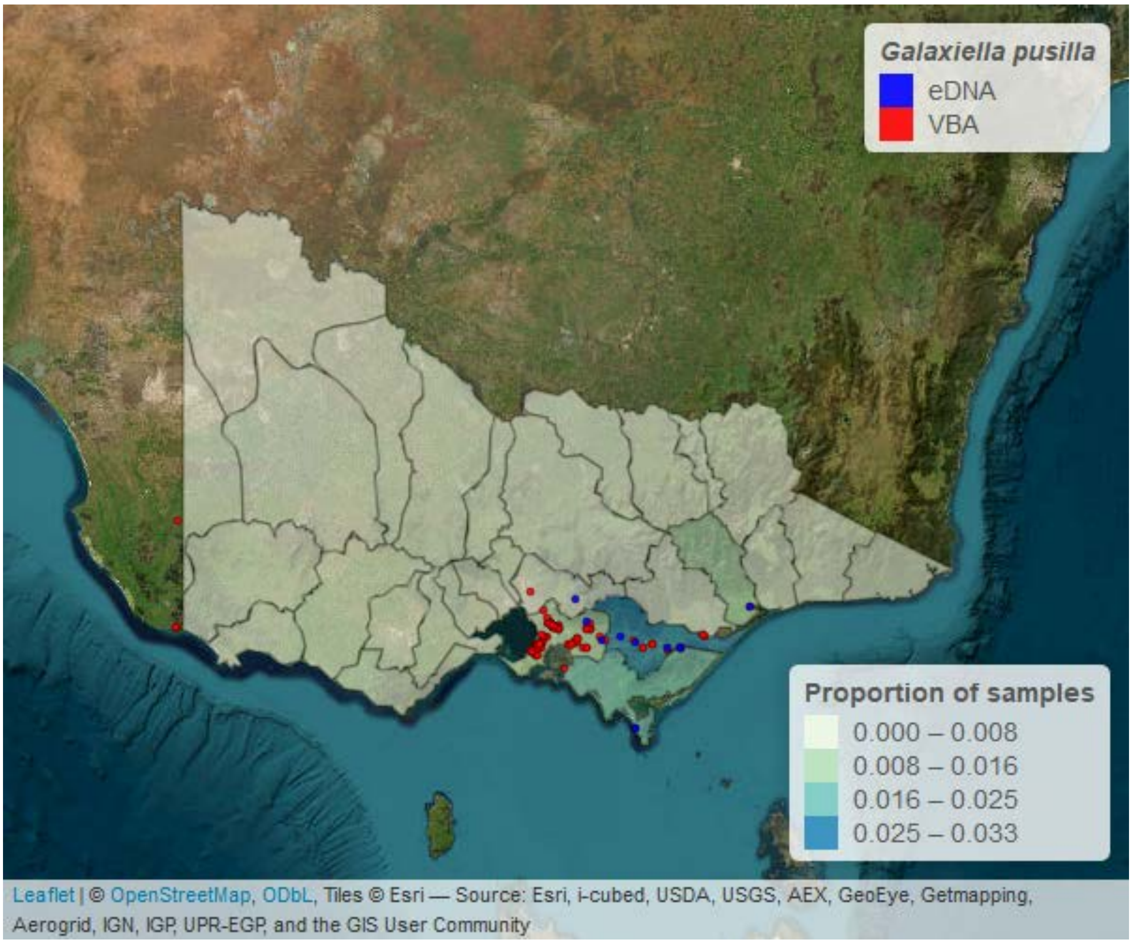

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Figure 15. Detections of *G. pusilla* with eDNA (blue dots) and those recorded in the VBA (red dots) for the last 20 years (2003-2023). Basins are colour coded for the proportion of eDNA detections positive for the species based on the number of sites sampled within the basin.

Flathead galaxias, *Galaxias rostratus* (FFG listed)

Galaxias rostratus occupies 2 river basins based on the records in the VBA. This species was detected in 1 river basin with eDNA sampling, which is known to have observed records according to VBA. *Galaxias rostratus* was detected in the following basin with eDNA sampling: Ovens River.

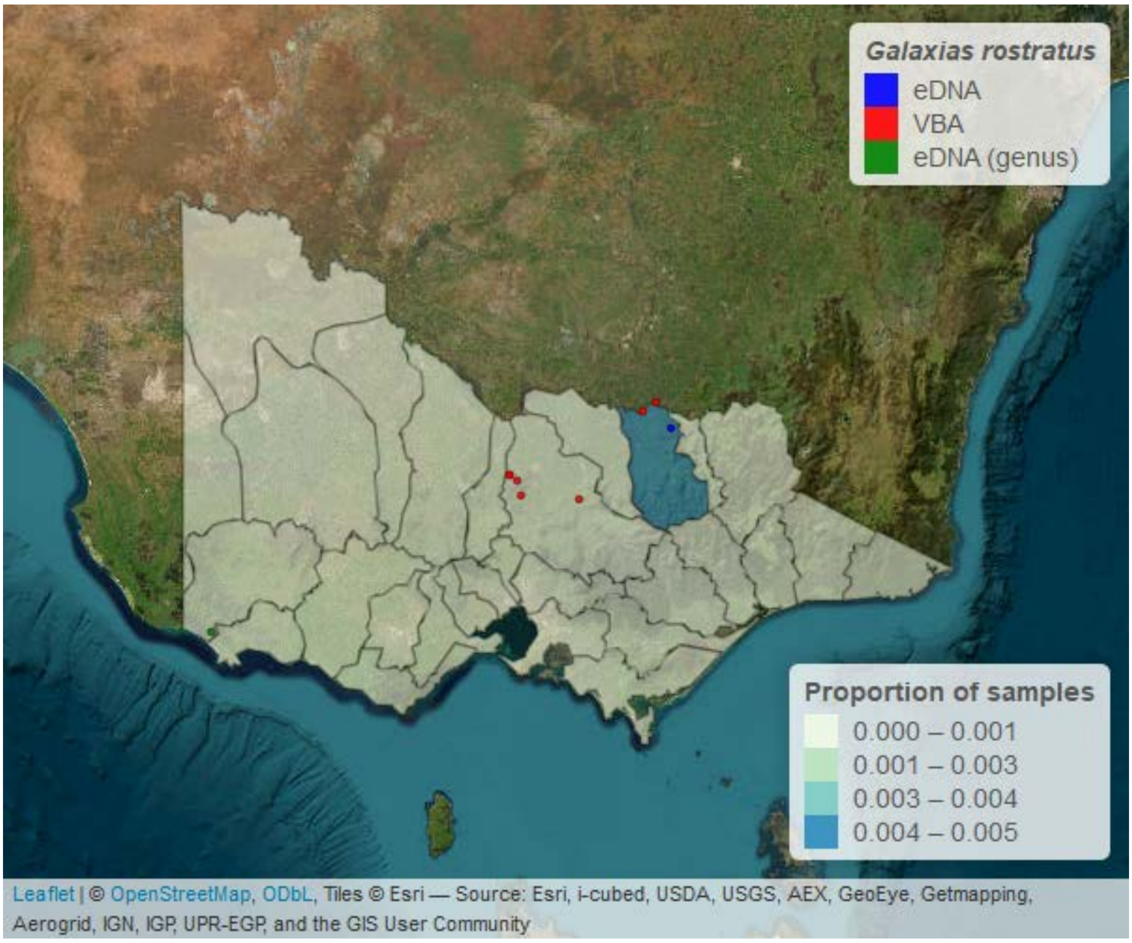

Figure 16. Detections of *G. rostratus* with eDNA (blue dots), eDNA detections at the genus level (green dots) and those recorded in the VBA (red dots) for the last 20 years (2003-2023). Basins are colour coded for the proportion of eDNA detections positive for the species based on the number of sites sampled within the basin.

Little galaxias, *Galaxiella toourtkoourt* (FFG listed)

Galaxiella toourtkoourt occupies 4 river basins based on the records in the VBA. This species was detected in 4 river basins with eDNA sampling, all 4 of which were observed previously according to VBA. *Galaxiella toourtkoourt* was detected in the following basins with eDNA sampling: Barwon River, Glenelg River, Hopkins River and Portland Coast.

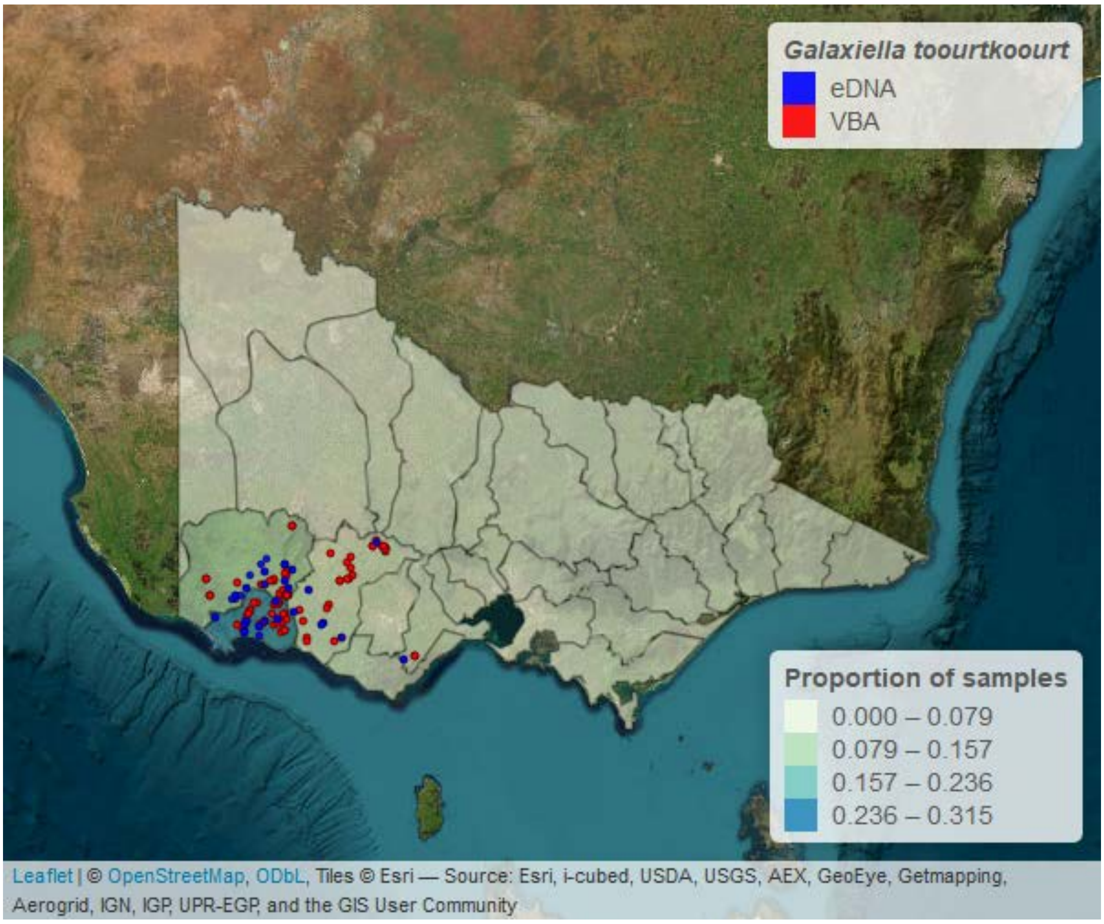

Figure 17. Detections of *G. toourtkoourt* with eDNA (blue dots) and those recorded in the VBA (red dots) for the last 20 years (2003-2023). Basins are colour coded for the proportion of eDNA detections positive for the species based on the number of sites sampled within the basin.

Cox's gudgeon, *Gobiomorphus coxii* (FFG listed)

Gobiomorphus coxii occupies 1 river basin based on the records in the VBA. This species was detected in 1 river basin with eDNA sampling, which is known to be observed previously according to VBA. *Gobiomorphus coxii* was detected in the following basins with eDNA sampling: Snowy River. There were three eDNA detections of *G. coxii*, all in the same river system (Buchan River) as the last known detection of the species in VBA, potential extending its known range. This warrants further investigation to elucidate the extent of its current range.

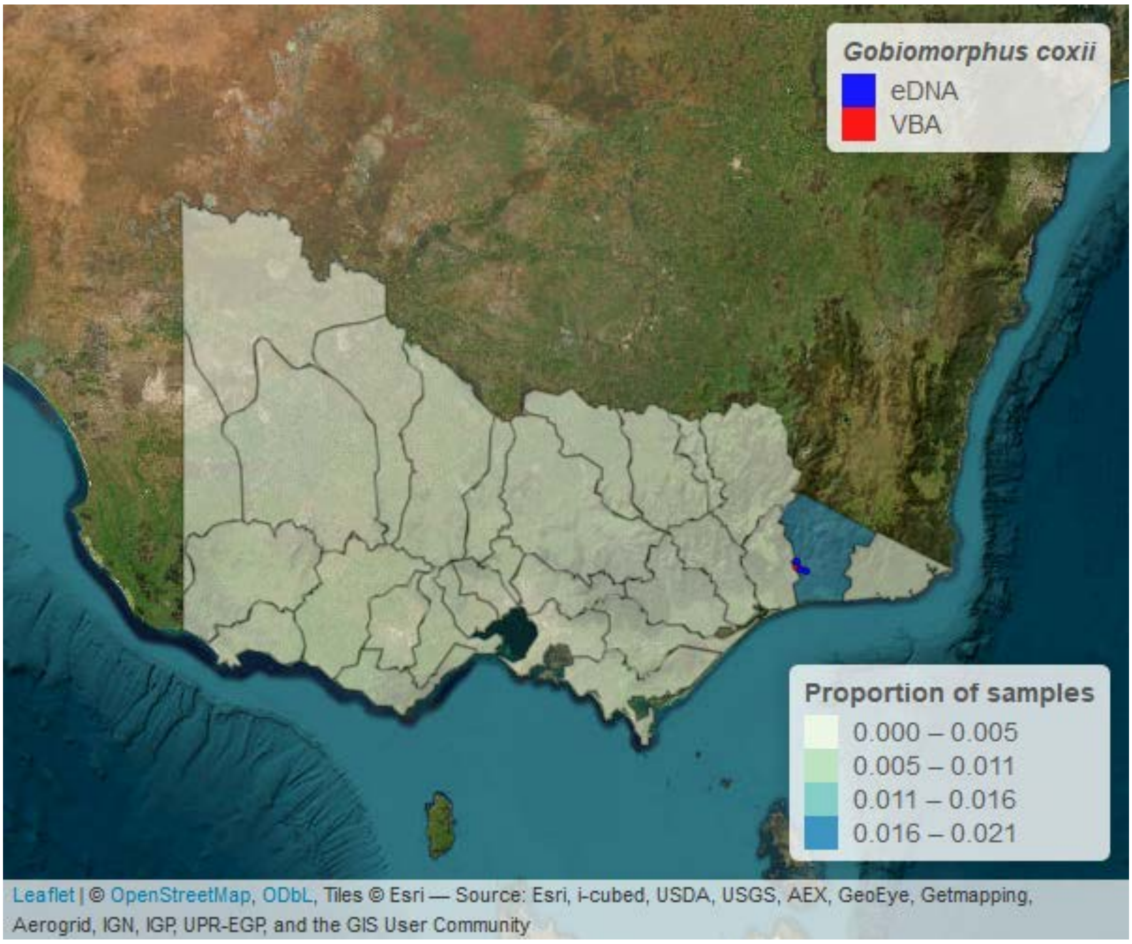

Figure 18. Detections of *G. coxii* with eDNA (blue dots) and those recorded in the VBA (red dots) for the last 20 years (2003-2023). Basins are colour coded for the proportion of eDNA detections positive for the species based on the number of sites sampled within the basin.

Carp gudgeons, *Hypseleotris* spp.

Hypseleotris spp. is a species complex comprising up to four different species that cannot be differentiated using the Vertebrate amplicon due to overlapping haplotypes. The species are also known to hybridise and co-occur, making identification through eDNA methods difficult. We therefore report on detections at the genus level. *Hypseleotris* spp. have been observed previously in 13 river basins according to VBA. This species was detected in 15 river basins with eDNA sampling, 11 of which were observed previously according to VBA. eDNA sampling therefore detected *Hypseleotris spp.* in 4 river basins for which there were no corresponding VBA records. These warrant further investigation, as they may extend the known range of *Hypseleotris* spp., or represent false positive site detections.

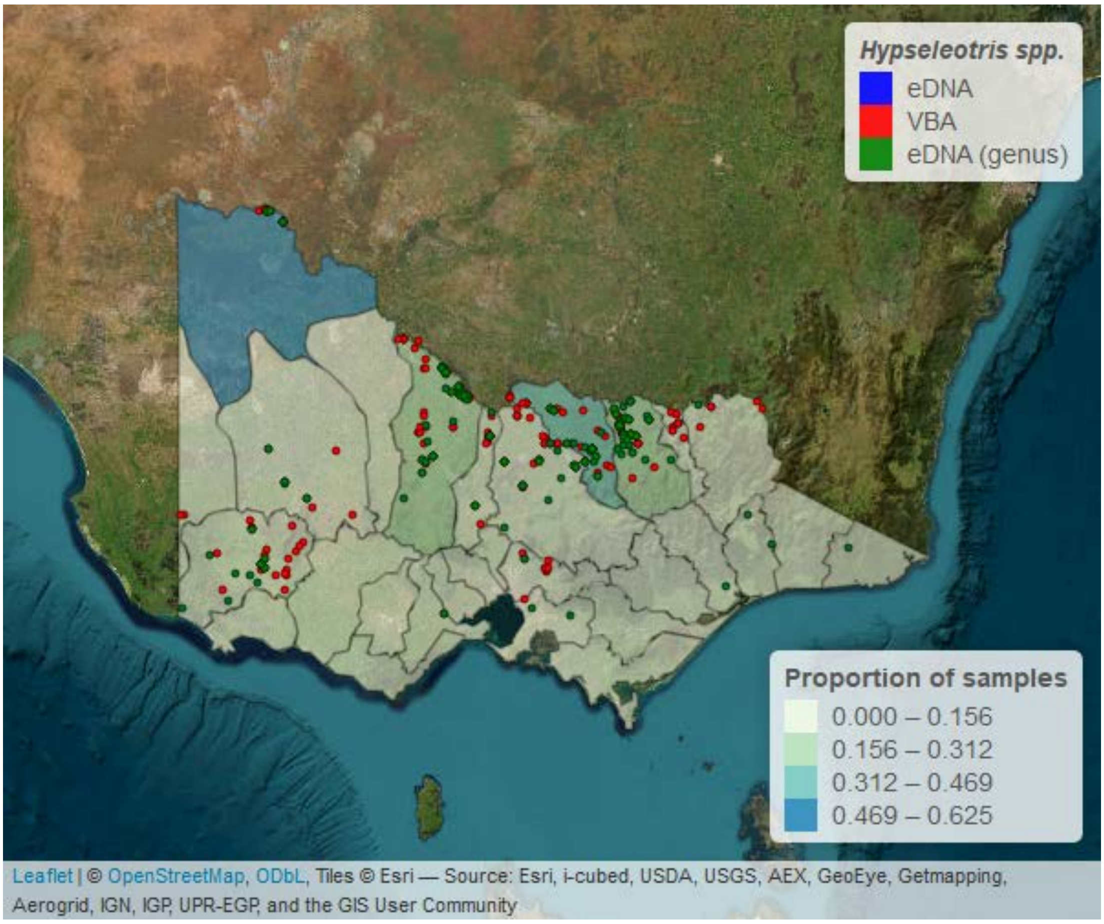

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Figure 19. Detections of *Hypseleotris* spp. with eDNA (green dots) and those recorded in the VBA (red dots) for the last 20 years (2003-2023). Basins are colour coded for the proportion of eDNA detections positive for the taxa based on the number of sites sampled within the basin.

Murray river rainbowfish, *Melanotaenia fluviatilis* (FFG listed)

Melanotaenia fluviatilis occupies 6 river basins based on the records in the VBA. This species was detected in 4 river basins with eDNA sampling, all 4 of which were observed previously according to VBA. *Melanotaenia fluviatilis* was detected in the following basins with eDNA sampling: Broken River, Campaspe River, Loddon River and Ovens River. The species is found in the Murray River in many of these basins, and given that there were few samples taken from the Murray River, this is likely why it was not detected in the Mallee and Goulburn River Basins.

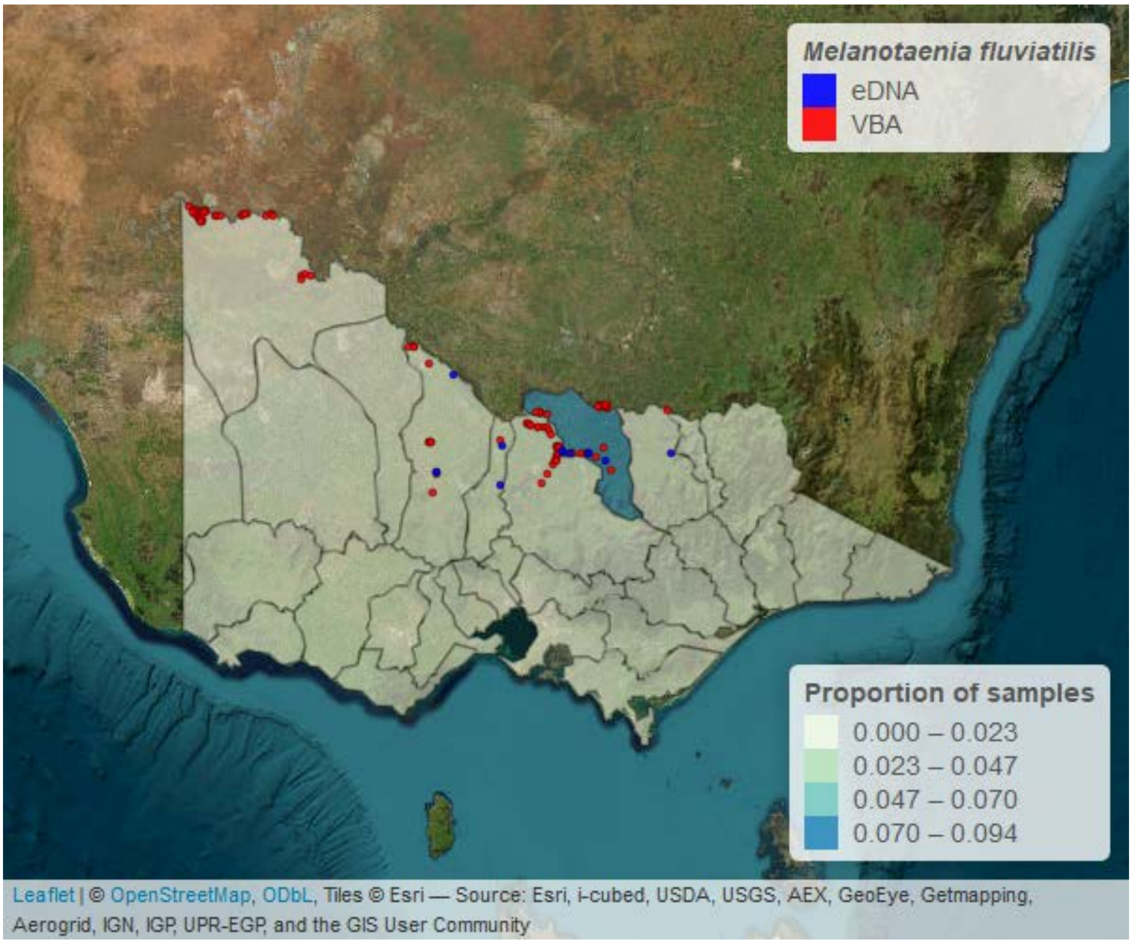

Figure 20. Detections of *M. fluviatilis* with eDNA (blue dots) and those recorded in the VBA (red dots) for the last 20 years (2003-2023). Basins are colour coded for the proportion of eDNA detections positive for the species based on the number of sites sampled within the basin.

Southern pygmy perch, *Nannoperca australis*

Nannoperca australis occupies 19 river basins based on the records in the VBA. This species was detected in 27 river basins with eDNA sampling, 18 of which were observed previously according to VBA. eDNA sampling therefore detected *Nannoperca australis* in 9 river basins for which there were no corresponding VBA records. The only basins in which *Nannoperca australis* was not detected with eDNA sampling are Mallee and Millicent Coast. We also provide eDNA detections that were stuck at the genus level (because they could not confidently be allocated to species) in Figure 21. Many of these detections are likely to represent *N. australis*, *N. obscura*, or *N.* sp. 1; all of which are closely related species and cannot be differentiated with the Vertebrate (or fish) amplicon(s).

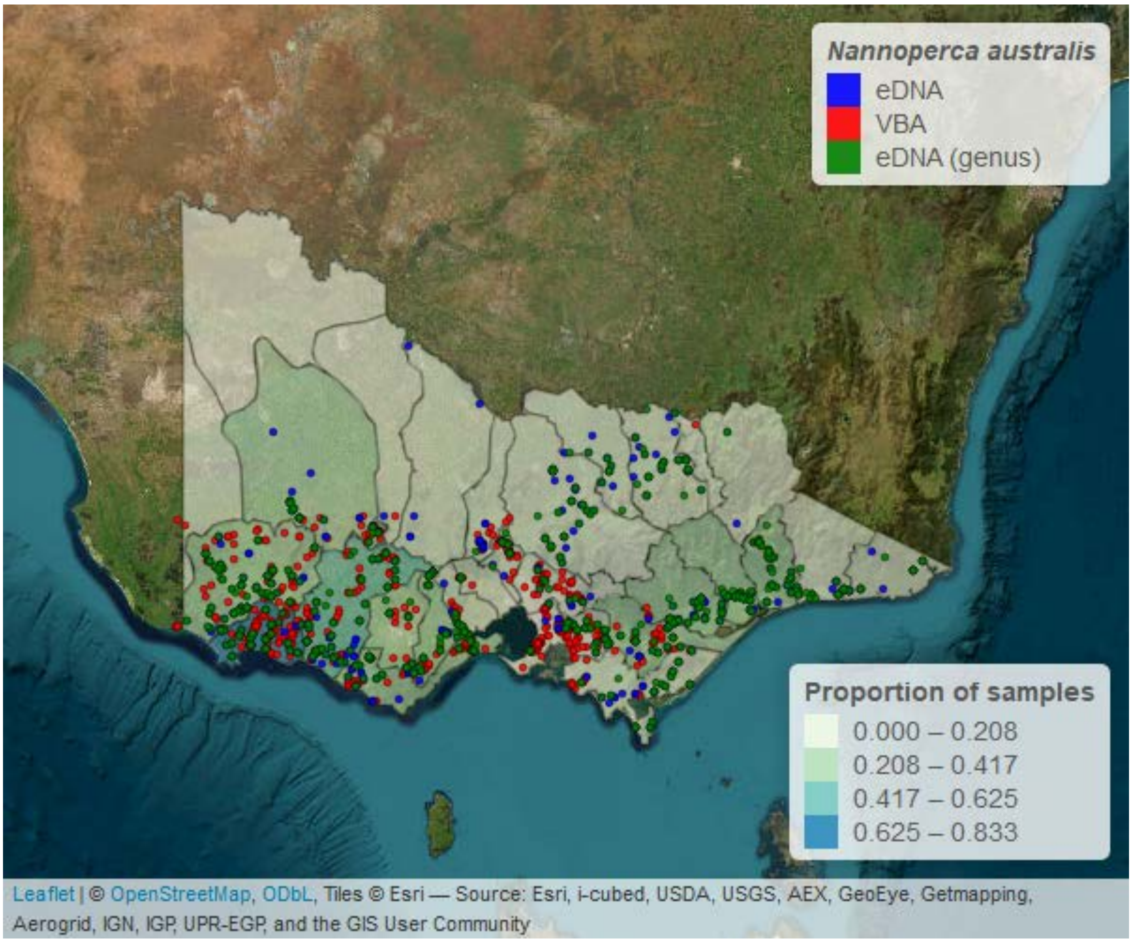

Figure 21. Detections of *N. australis* with eDNA (blue dots), eDNA detections at the genus level (green dots) and those recorded in the VBA (red dots) for the last 20 years (2003-2023). Basins are colour coded for the proportion of eDNA detections positive for the species based on the number of sites sampled within the basin.

Variegated pygmy perch, *Nannoperca variegata* (FFG listed)

Nannoperca variegata occupies 1 river basin based on the records in the VBA. This species was detected in 1 river basin with eDNA sampling, which is the same river basin with observed records according to VBA. *Nannoperca variegata* was detected in the following basin with eDNA sampling: Glenelg River. Again, we provide detections at the genus level in Figure 22, some of which may be *N. variegate* (particular those in the Glenelg River basin).

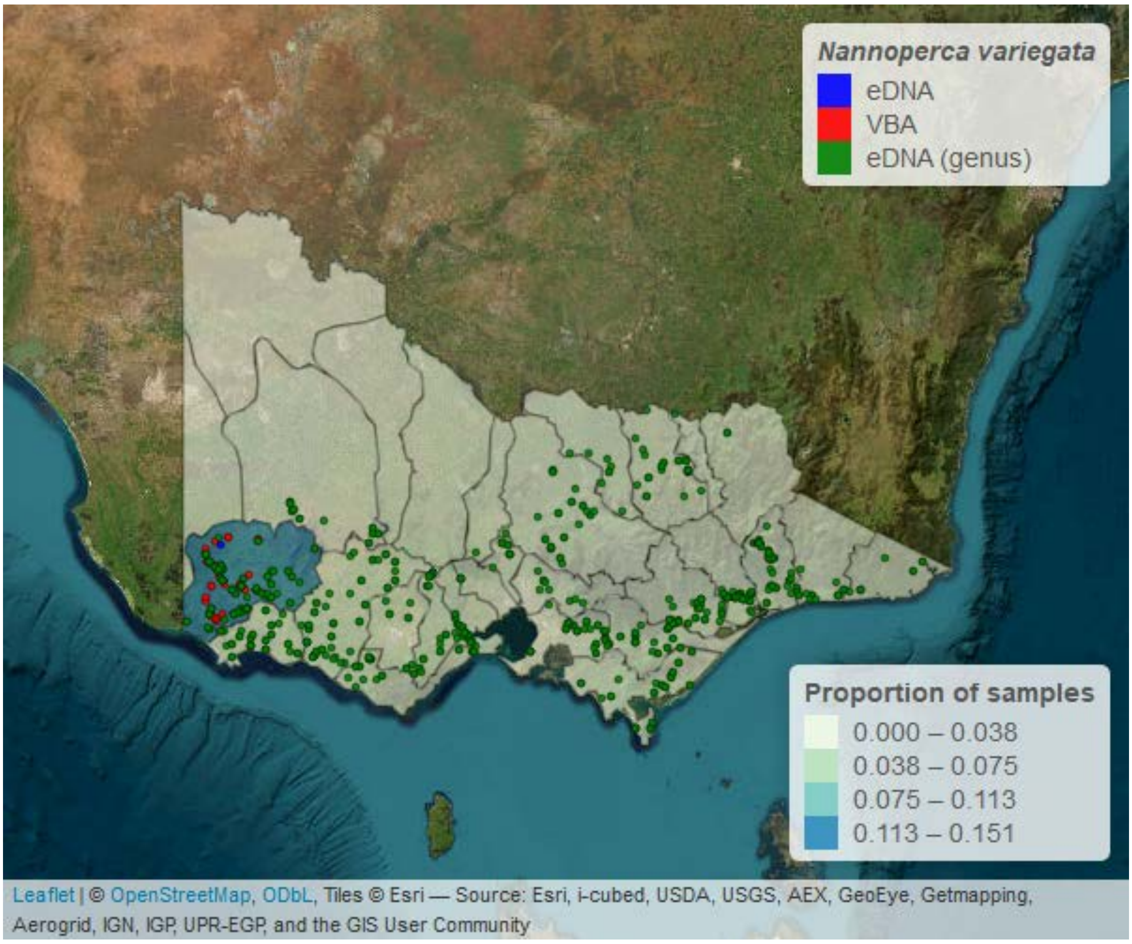

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Figure 22. Detections of *N. variegata* with eDNA (blue dots), eDNA detections at the genus level (green dots) and those recorded in the VBA (red dots) for the last 20 years (2003-2023). Basins are colour coded for the proportion of eDNA detections positive for the species based on the number of sites sampled within the basin.

Bony bream, *Nematalosa erebi*

Nematalosa erebi occupies 4 river basins based on the records in the VBA. This species was detected in 4 river basins with eDNA sampling, 3 of which were observed previously according to VBA records. eDNA sampling therefore detected *Nematalosa erebi* in 1 river basin for which there were no corresponding VBA records. *Nematalosa erebi* was detected in the following basins with eDNA sampling: Broken River, Loddon River, Mallee and Yarra River. Few eDNA samples in the Murray River is the likely reason why *N. erebi* wasn't detected in Broken River Basin, and only a few detections in the Mallee Basin. There is a potential false positive site detection in the Yarra River Basin.

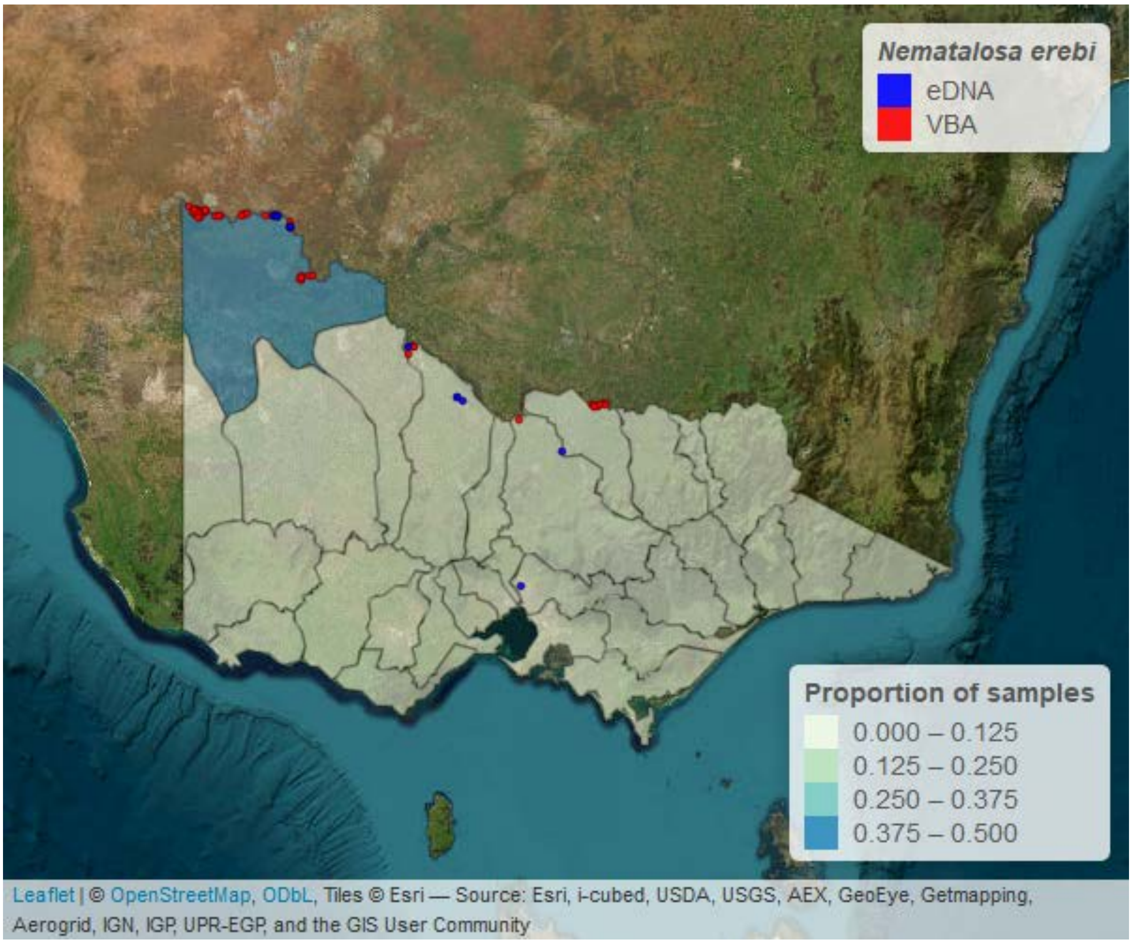

Figure 23. Detections of *N. erebi* with eDNA (blue dots) and those recorded in the VBA (red dots) for the last 20 years (2003-2023). Basins are colour coded for the proportion of eDNA detections positive for the species based on the number of sites sampled within the basin.

Australian mudfish, *Neochanna cleaveri* (FFG listed)

Neochanna cleaveri occupies 2 river basins based on the records in the VBA. This species was detected in 1 river basin with eDNA sampling, which was not previously recorded in the VBA from the last 20 years. *Neochanna cleaveri* was detected in the following basin with eDNA sampling: South Gippsland. The species has been historically in this basin, with the last records from a similar location as the eDNA detections (Wilsons Promontory) in 2002 (over 20 years ago). The species is listed under the FFG Act 1988, and therefore these represent significant detections worthy of further investigation.

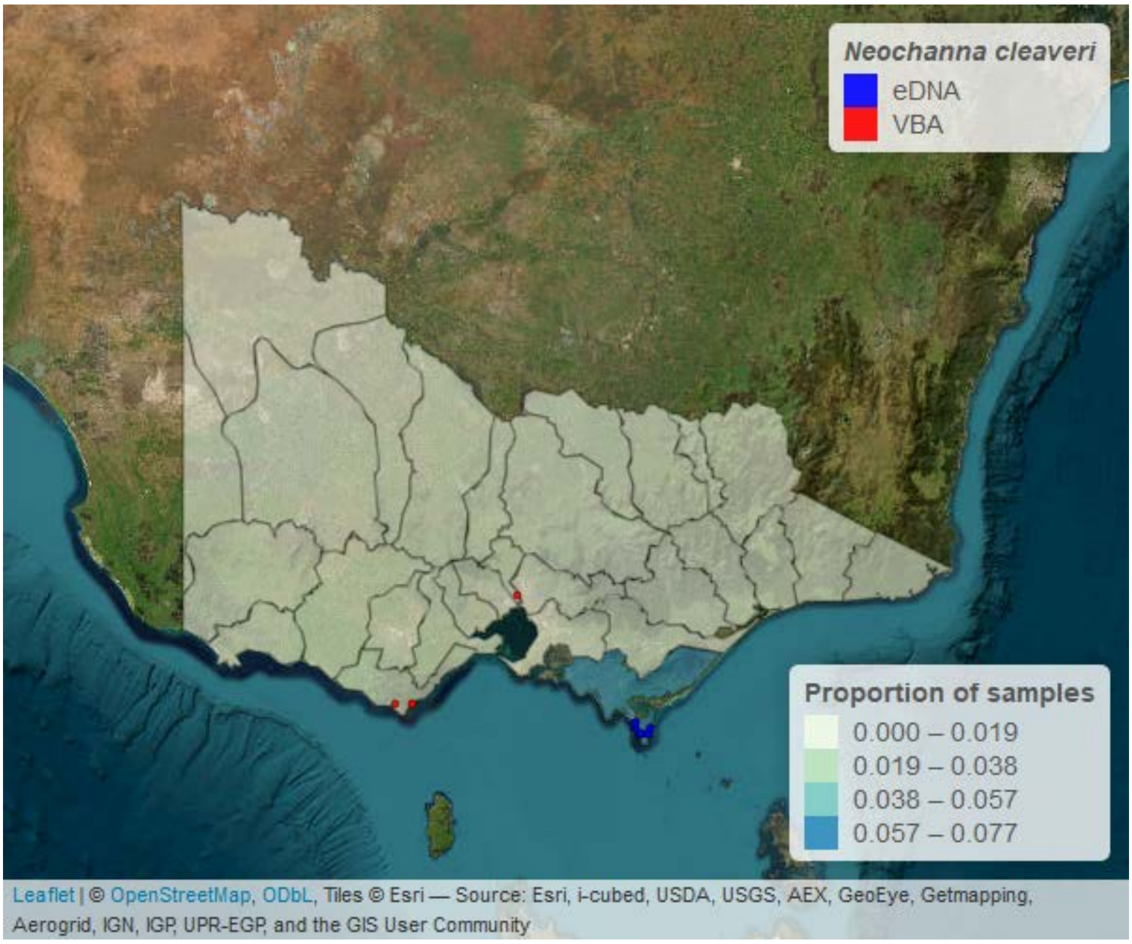

Figure 24. Detections of *N. cleaveri* with eDNA (blue dots) and those recorded in the VBA (red dots) for the last 20 years (2003-2023). Basins are colour coded for the proportion of eDNA detections positive for the species based on the number of sites sampled within the basin.

Flathead gudgeon, *Philypnodon grandiceps*

Philypnodon grandiceps is a widespread species, occupying 28 river basins based on the records in the VBA. This species was detected in 27 river basins with eDNA sampling, 26 of which were observed previously according to VBA. eDNA sampling therefore detected *Philypnodon grandiceps* in 1 river basin (Tambo River Basin) for which there were no corresponding VBA records in the last 20 years. The only basins in which *Philypnodon grandiceps* was not detected with eDNA sampling are Broken River and Millicent Coast.

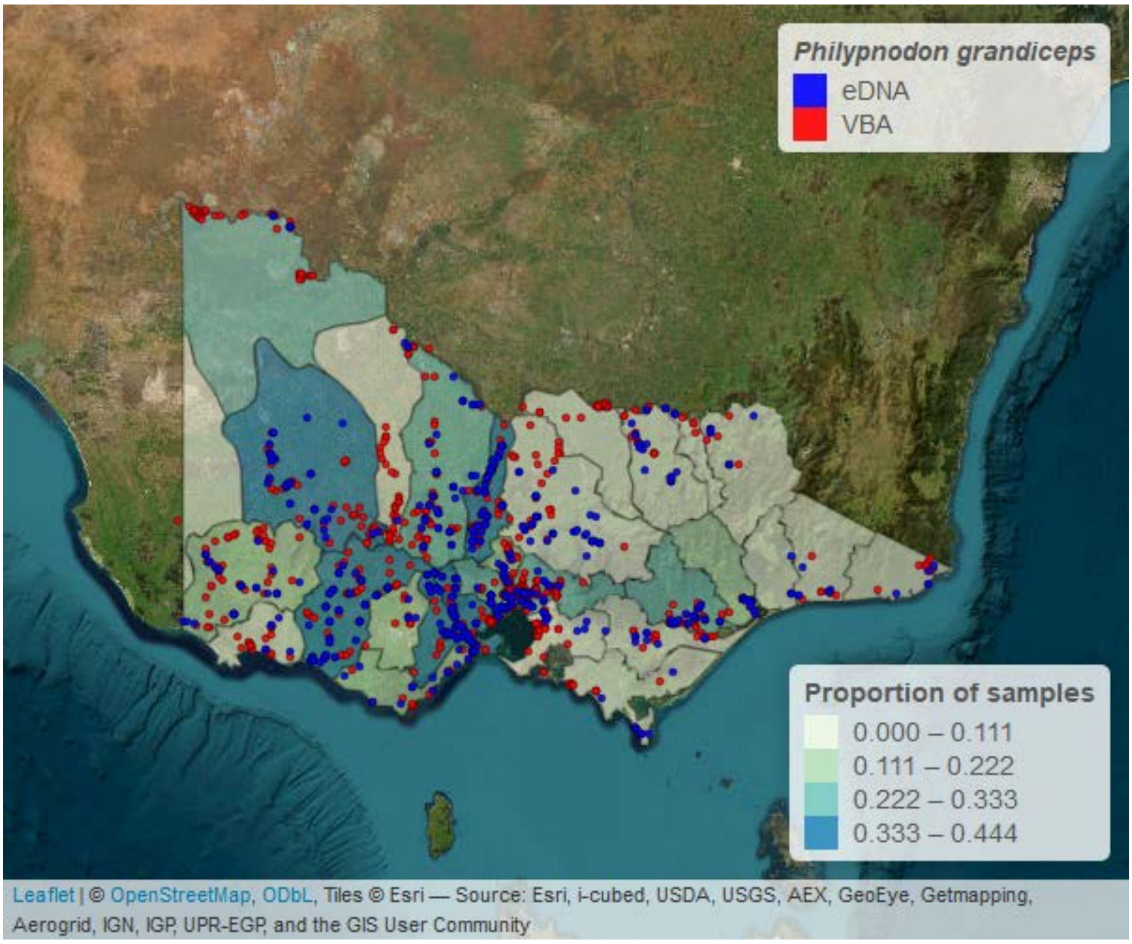

Figure 25. Detections of *P. grandiceps* with eDNA (blue dots) and those recorded in the VBA (red dots) for the last 20 years (2003-2023). Basins are colour coded for the proportion of eDNA detections positive for the species based on the number of sites sampled within the basin.

Dwarf flathead gudgeon, *Philypnodon macrostomus*

Philypnodon macrostomus occupies 6 river basins based on the records in the VBA. This species was detected in 9 river basins with eDNA sampling, 5 of which were observed previously according to VBA. eDNA sampling therefore detected *Philypnodon macrostomus* in 4 river basins for which there were no corresponding VBA records. Some of these eDNA detections are single detections from a basin, and therefore could represent false positive site detections (e.g. Goulburn River, Latrobe River and South Gippsland Basins).

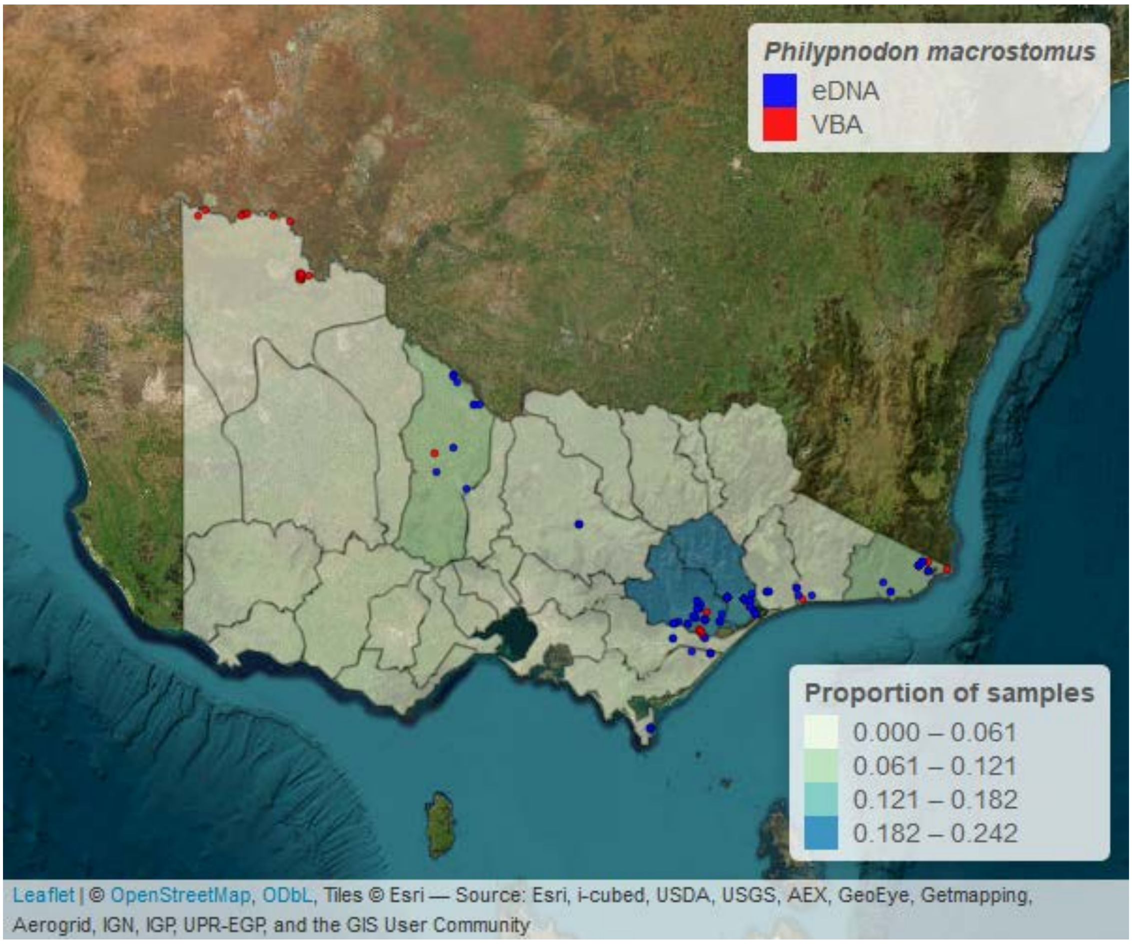

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Figure 26. Detections of *P. macrostomus* with eDNA (blue dots) and those recorded in the VBA (red dots) for the last 20 years (2003-2023). Basins are colour coded for the proportion of eDNA detections positive for the species based on the number of sites sampled within the basin.

Australian grayling, *Prototroctes maraena* (FFG listed)

Prototroctes maraena occupies 12 river basins based on the records in the VBA. This species was detected in 3 river basins with eDNA sampling, all 3 of which were observed previously according to VBA. *Prototroctes maraena* was detected in the following basins with eDNA sampling: Otway Coast, Thomson River and Yarra River.

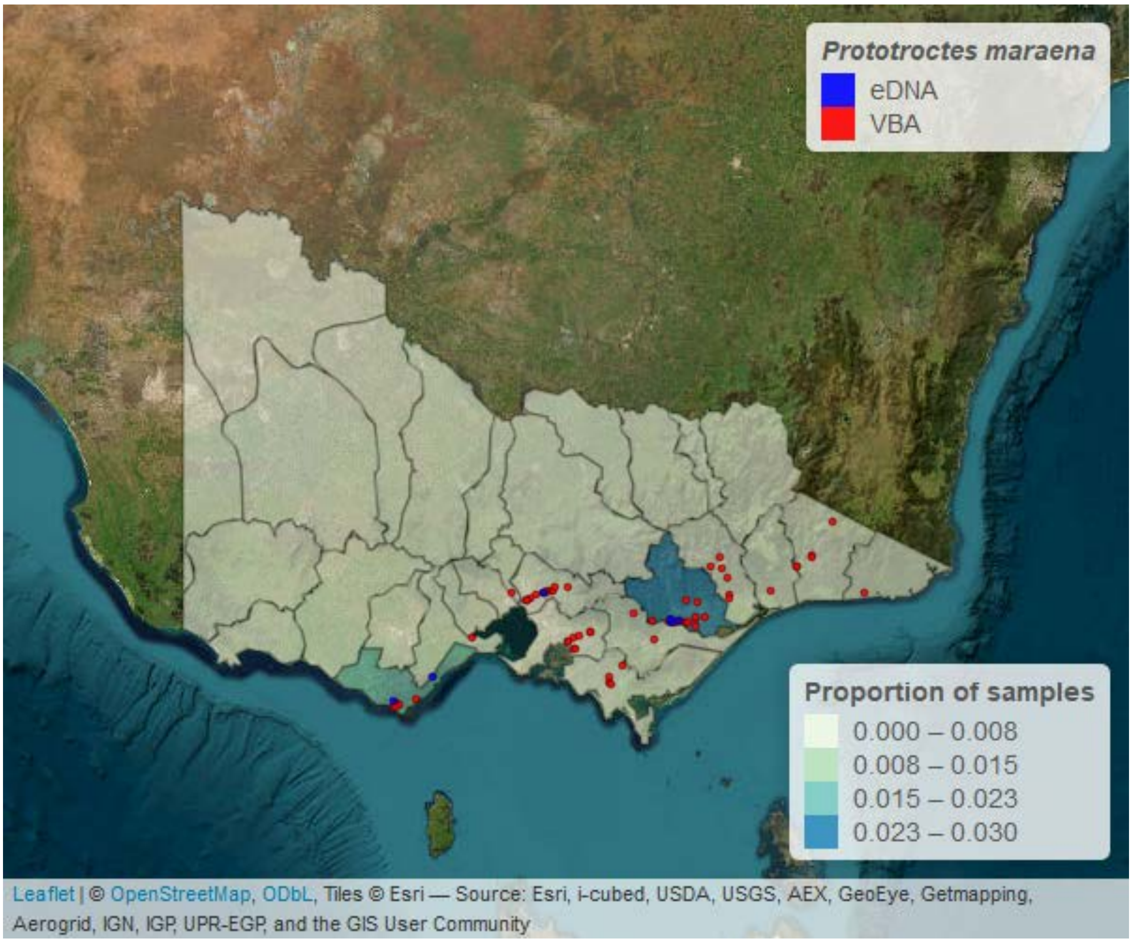

Figure 27. Detections of *P. maraena* with eDNA (blue dots) and those recorded in the VBA (red dots) for the last 20 years (2003-2023). Basins are colour coded for the proportion of eDNA detections positive for the species based on the number of sites sampled within the basin.

Australian smelt, *Retropinna semoni*

Retropinna semoni is a widespread species, occupying 28 river basins based on the records in the VBA. This species was detected in 26 river basins with eDNA sampling, all 26 of which were observed previously according to VBA. The only basins in which *Retropinna semoni* was not detected with eDNA sampling are Avoca River, Millicent Coast and Portland Coast. The absence of Avoca River Basin detections could be explained by a lack of eDNA sampling sites in the Avoca River, where all the VBA records are from.

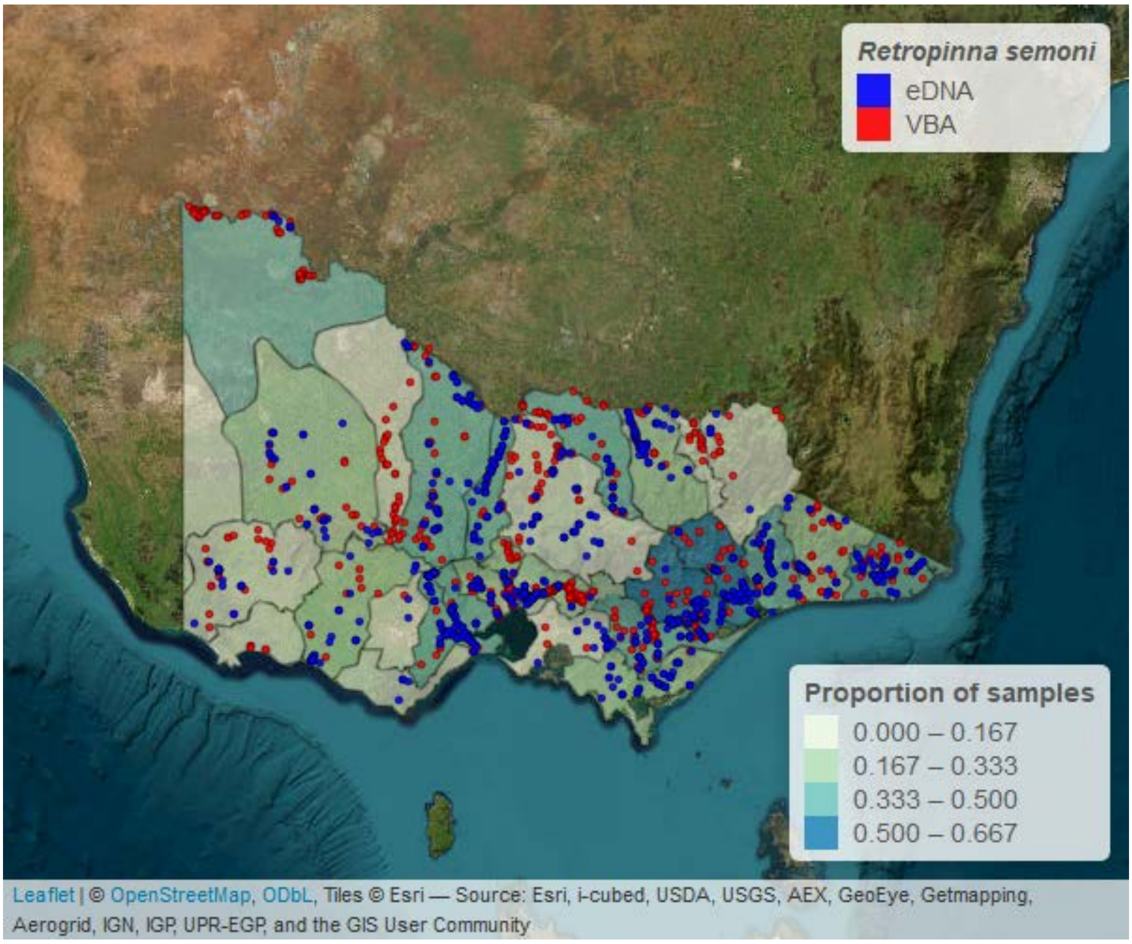

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Figure 28. Detections of *R. semoni* with eDNA (blue dots) and those recorded in the VBA (red dots) for the last 20 years (2003-2023). Basins are colour coded for the proportion of eDNA detections positive for the species based on the number of sites sampled within the basin.

Eel-tailed catfish, *Tandanus tandanus* (FFG listed)

Tandanus tandanus occupies 7 river basins based on the records in the VBA. This species was detected in 6 river basins with eDNA sampling, 3 of which were observed previously according to VBA. eDNA sampling therefore detected *Tandanus tandanus* in 3 river basins for which there were no corresponding VBA records. There were only single eDNA detections in each of these basins, and therefore follow up is required to determine whether they are real site detections for this species or are potentially false positive site detections.

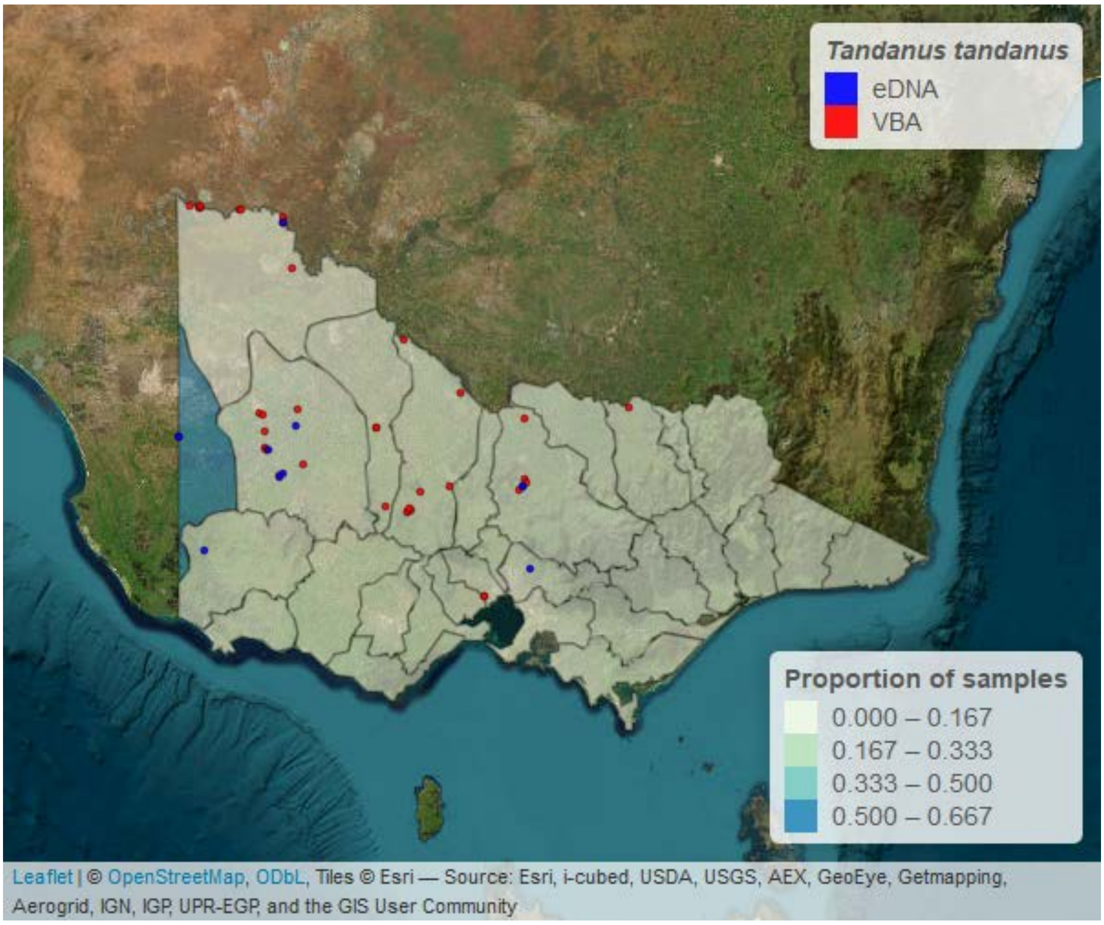

Figure 29. Detections of *T. tandanus* with eDNA (blue dots) and those recorded in the VBA (red dots) for the last 20 years (2003-2023). Basins are colour coded for the proportion of eDNA detections positive for the species based on the number of sites sampled within the basin.

Coastal species

Climbing galaxias, *Galaxias brevipinnis*

Galaxias brevipinnis occupies 17 river basins based on the records in the VBA. This species was detected in 21 river basins with eDNA sampling, 15 of which were observed previously according to VBA. eDNA sampling therefore detected *Galaxias brevipinnis* in 6 river basins for which there were no corresponding VBA records. This is a surprising level of detections in basins for which there are no VBA records (particularly the Goulburn River Basin) and may represent some false positive sample detections due to inadequate reference sequences.

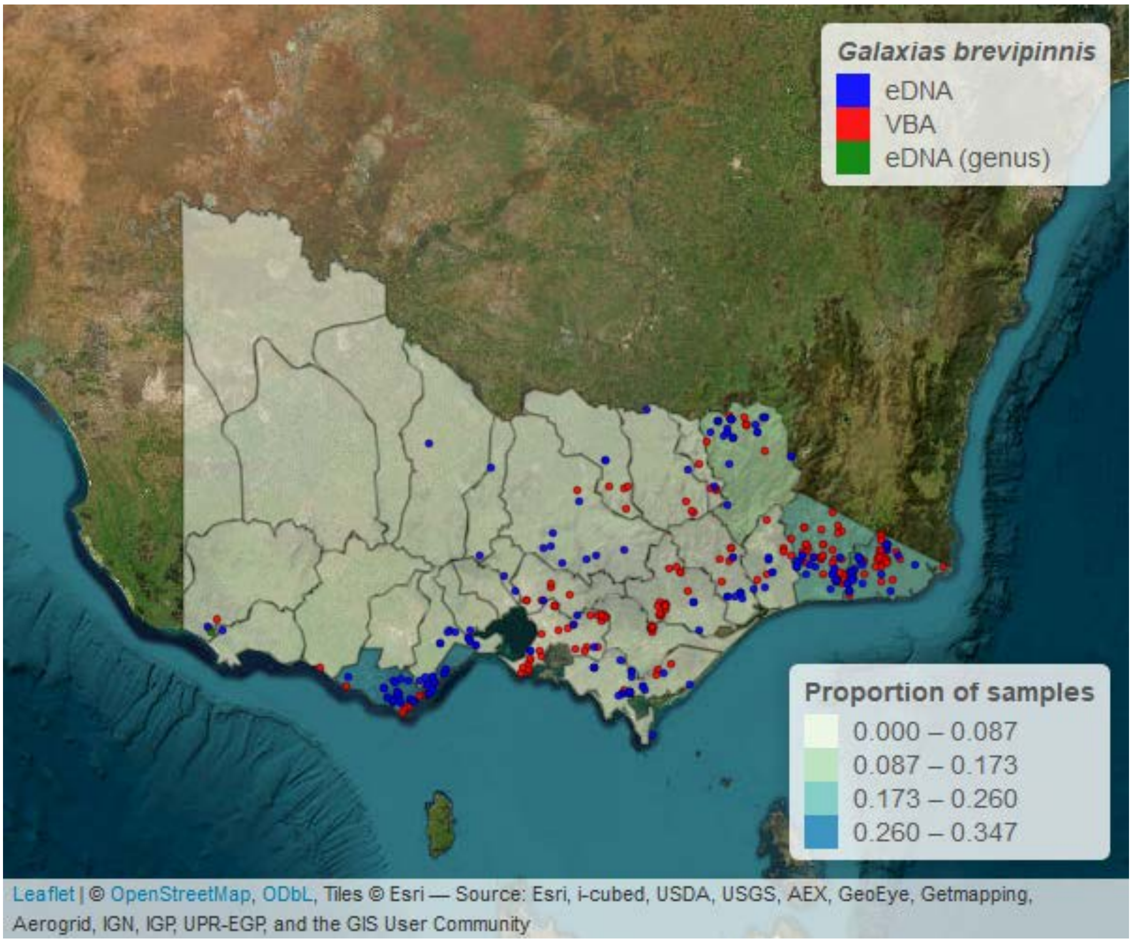

Figure 30. Detections of *G. brevipinnis* with eDNA (blue dots), eDNA detections at the genus level (green dots) and those recorded in the VBA (red dots) for the last 20 years (2003-2023). Basins are colour coded for the proportion of eDNA detections positive for the species based on the number of sites sampled within the basin.

Common galaxias, *Galaxias maculatus*

Galaxias maculatus is a widespread species, occupying 22 river basins based on the records in the VBA. This species was detected in 24 river basins with eDNA sampling, 22 of which were observed previously according to VBA. eDNA sampling therefore detected *Galaxias maculatus* in 2 river basins for which there were no corresponding VBA records. These may represent false positive site detections and require further investigation.

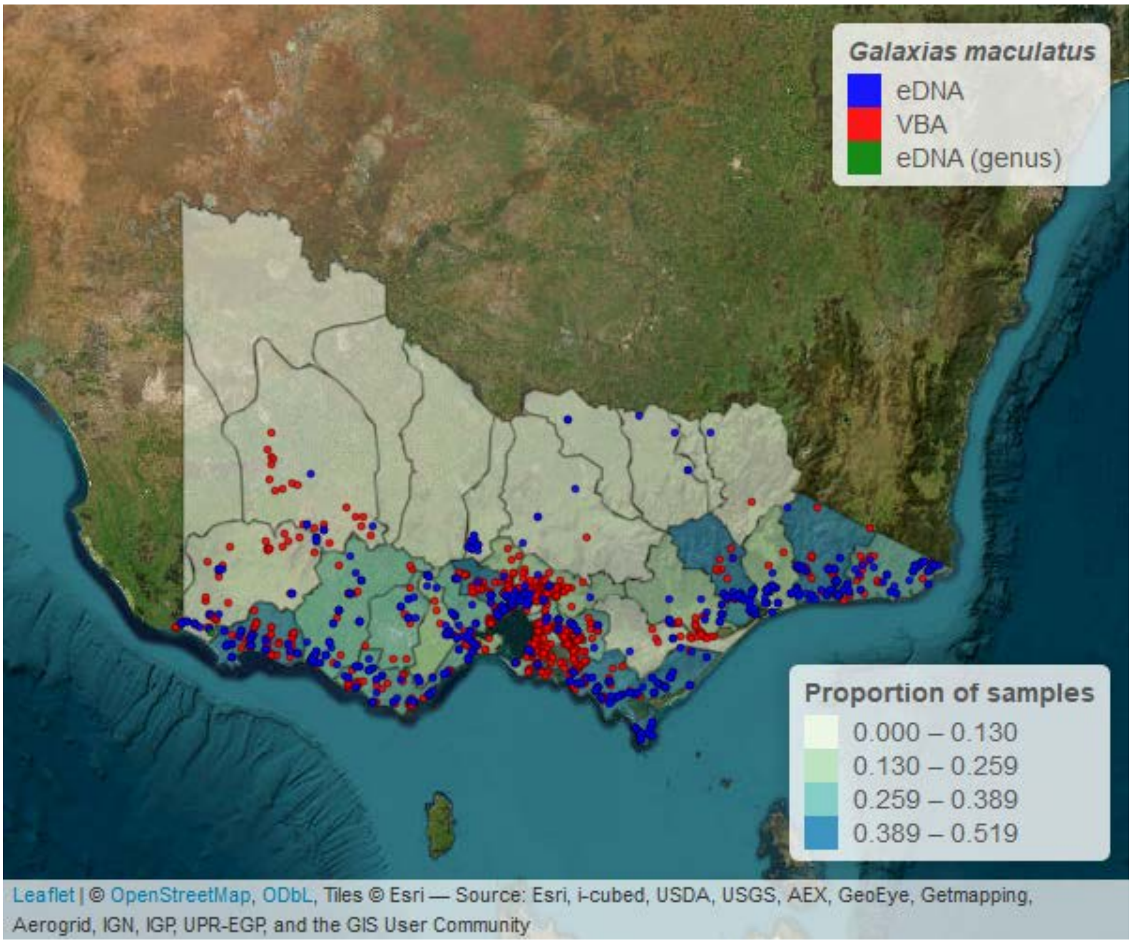

Figure 31. Detections of *G. maculatus* with eDNA (blue dots), eDNA detections at the genus level (green dots) and those recorded in the VBA (red dots) for the last 20 years (2003-2023). Basins are colour coded for the proportion of eDNA detections positive for the species based on the number of sites sampled within the basin.

Spotted galaxias, *Galaxias truttaceus*

Galaxias truttaceus occupies 16 river basins based on the records in the VBA. This species was detected in only 8 river basins with eDNA sampling, all 8 of which were observed previously according to VBA. It is surprising how few detections were found for this species, and this may represent inadequate reference sequences both within and between *Galaxias* species. Some detections were also found in the Campaspe River basin for which there are no recent detections in VBA (although there are detections >20 years ago in VBA). The number of site detections suggests that they are possibly real site detections.

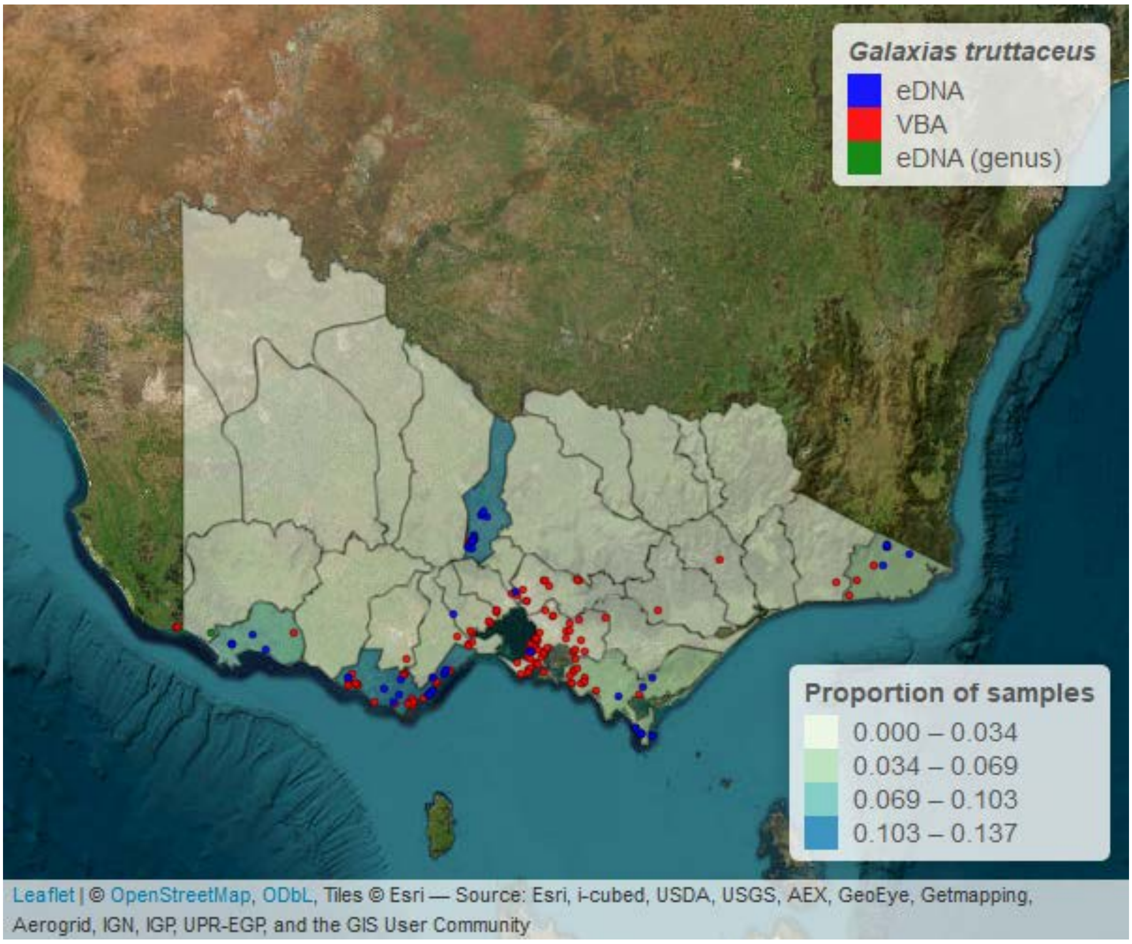

Figure 32. Detections of *G. truttaceus* with eDNA (blue dots), eDNA detections at the genus level (green dots) and those recorded in the VBA (red dots) for the last 20 years (2003-2023). Basins are colour coded for the proportion of eDNA detections positive for the species based on the number of sites sampled within the basin.

Estuary perch, *Macquaria colonorum*

Macquaria colonorum occupies 10 river basins based on the records in the VBA. This species was detected in 9 river basins with eDNA sampling, 5 of which were observed previously according to VBA. eDNA sampling therefore detected *Macquaria colonorum* in 4 river basins for which there were no corresponding VBA records. These were all coastal basins, and are therefore likely real detections.

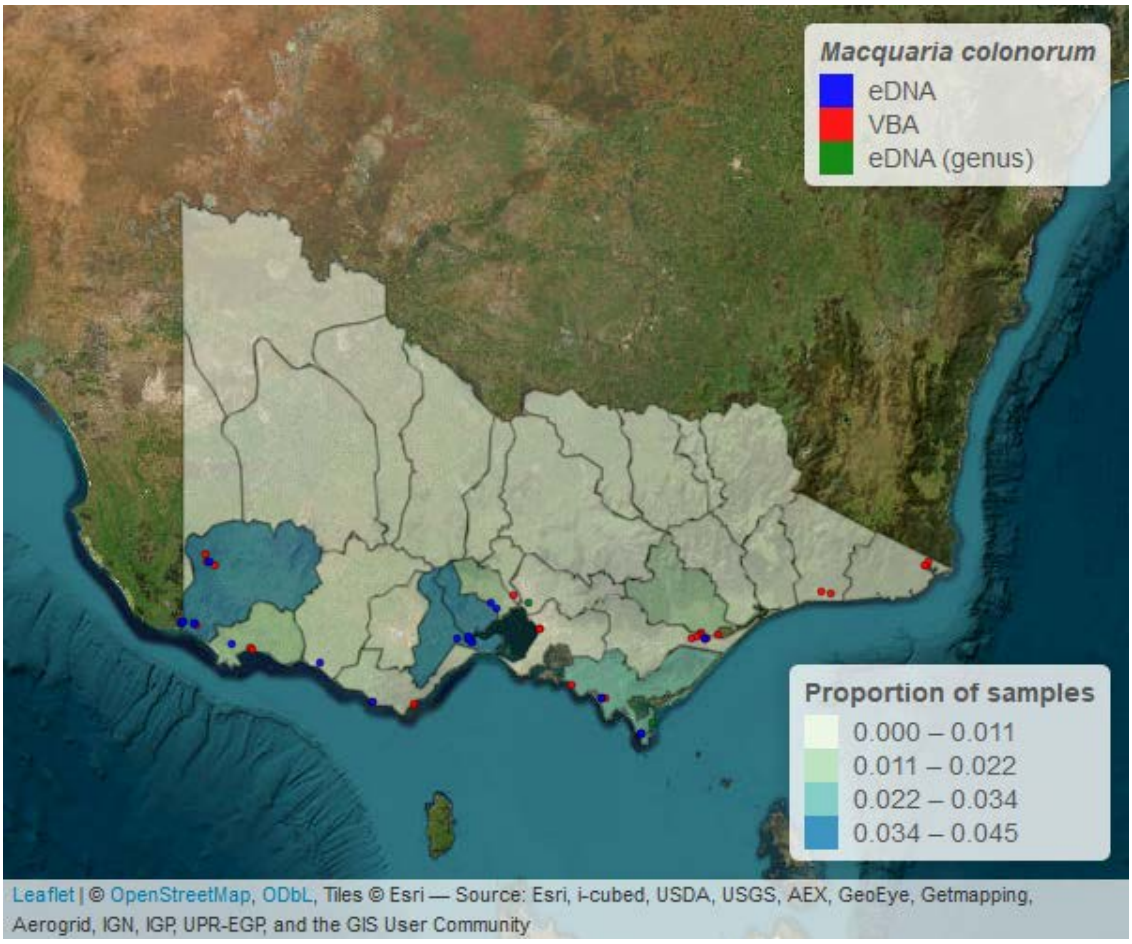

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Figure 33. Detections of *M. colonorum* with eDNA (blue dots), eDNA detections at the genus level (green dots) and those recorded in the VBA (red dots) for the last 20 years (2003-2023). Basins are colour coded for the proportion of eDNA detections positive for the species based on the number of sites sampled within the basin.

Australian bass, *Macquaria novemaculeata*

Macquaria novemaculeata was detected in 5 river basins with eDNA sampling. There was no corresponding data in the VBA database, however a review of the Atlas of Living Australia (ALA) indicated that they have been recorded as being present in 7 river basins (all east of Wilsons Promontory, not including VFA stocking programs). Both *M. colonorum* and *M. novemaculeata* are closely related species that hybridise and therefore further work is needed to determine whether they share ASVs.

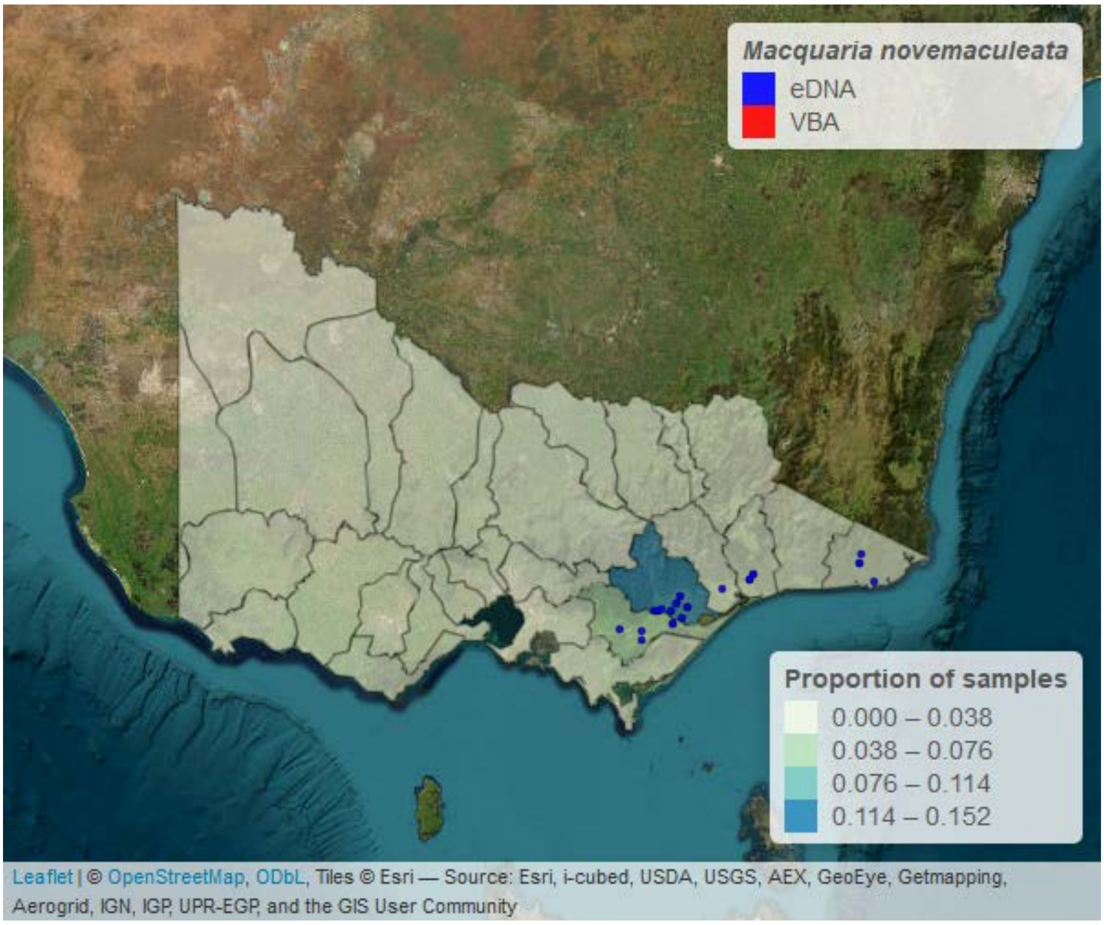

Figure 34. Detections of *M. novemaculeata* with eDNA (blue dots). Basins are colour coded for the proportion of eDNA detections positive for the species based on the number of sites sampled within the basin.

Flatback mangrove goby, *Mugilogobius platynotus* (FFG listed)

Mugilogobius platynotus occupies 2 river basins based on the records in the VBA. This species was detected in 1 river basin with eDNA sampling, which was observed previously according to VBA records. *Mugilogobius platynotus* was detected in the following basins with eDNA sampling: Bunyip River.

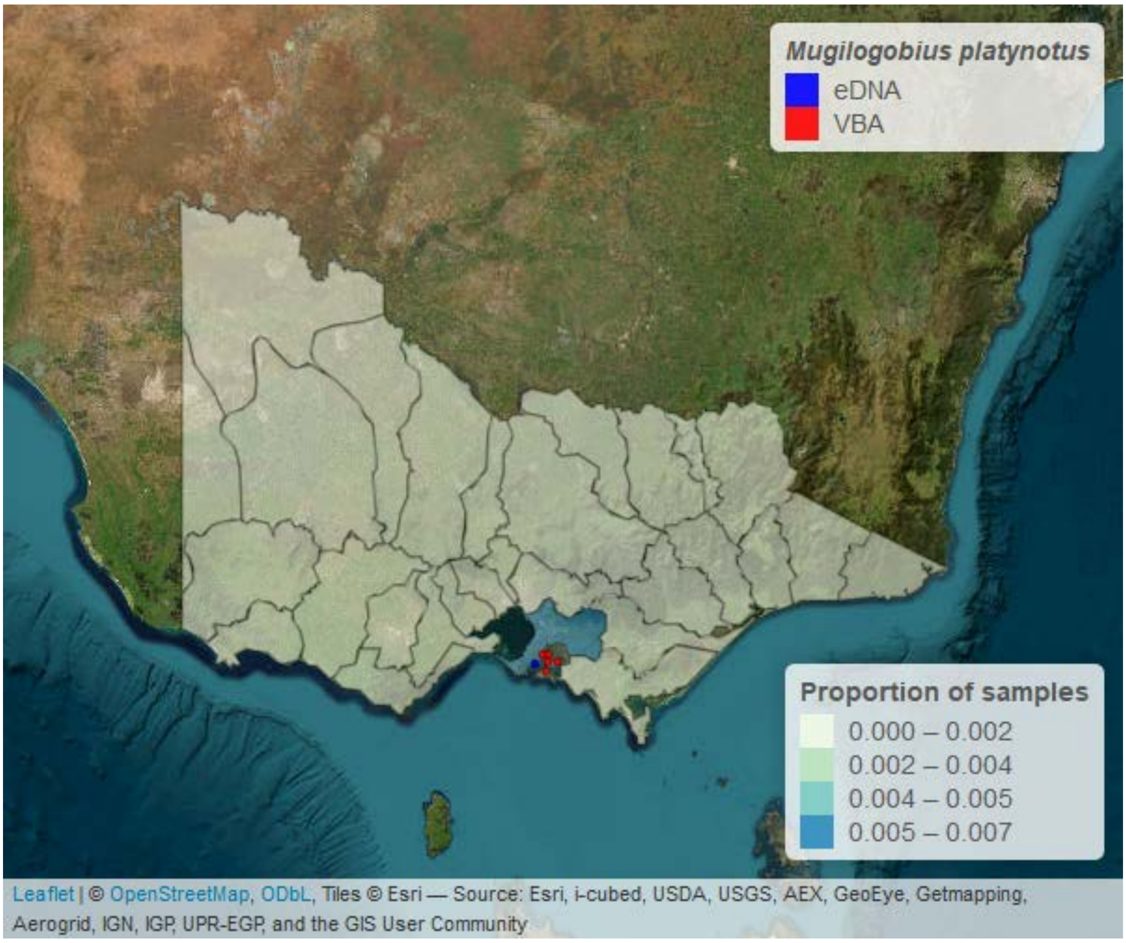

Figure 35. Detections of *Mugilogobius platynotus* with eDNA (blue dots) and those recorded in the VBA (red dots) for the last 20 years (2003-2023). Basins are colour coded for the proportion of eDNA detections positive for the species based on the number of sites sampled within the basin.

Invasive species

Goldfish, *Carassius auratus*

Carassius auratus is a widespread species, occupying 25 river basins based on the records in the VBA. This species was detected in 24 river basins with eDNA sampling, 23 of which were observed previously according to VBA. eDNA sampling therefore detected *Carassius auratus* in 1 river basin for which there were no corresponding VBA records (Tambo River). This was only a single detection and could potentially be a false positive site detection.

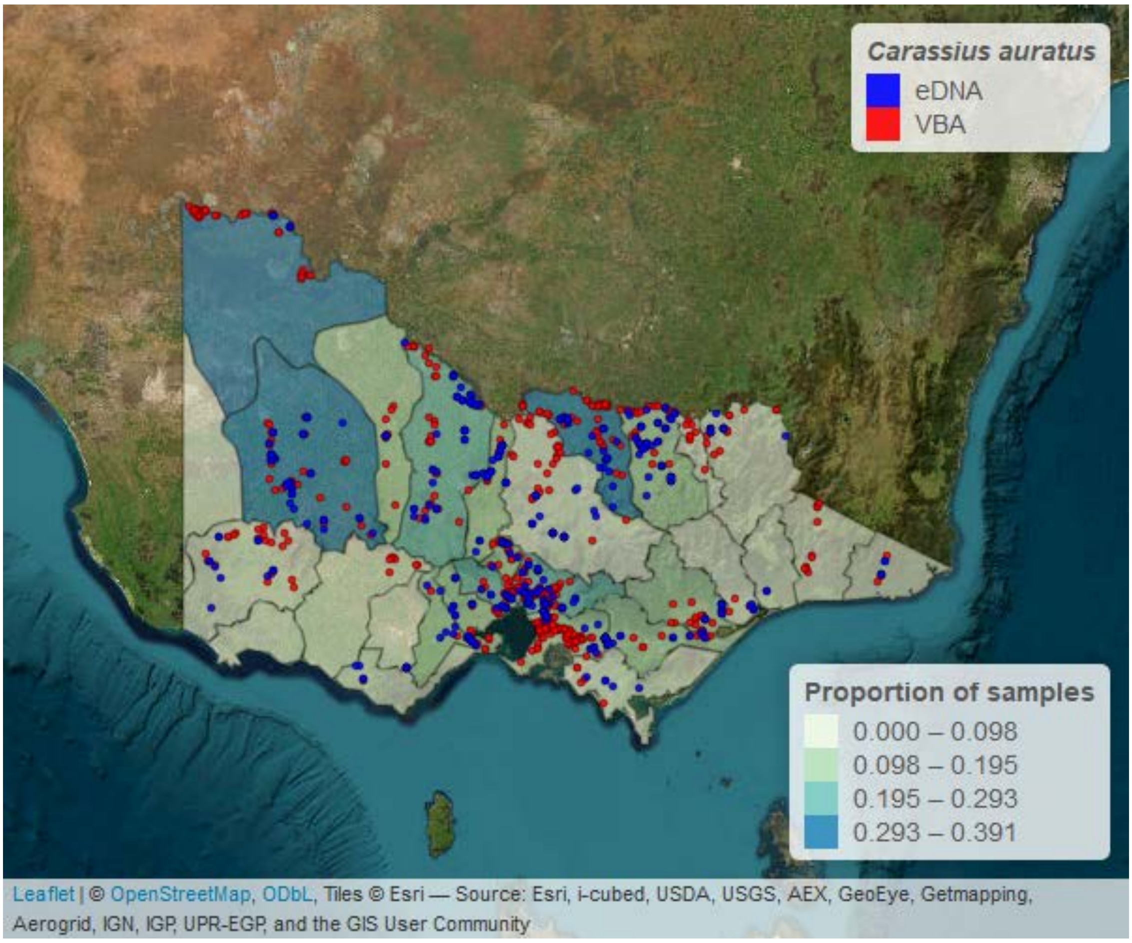

Figure 36. Detections of *C. auratus* with eDNA (blue dots) and those recorded in the VBA (red dots) for the last 20 years (2003-2023). Basins are colour coded for the proportion of eDNA detections positive for the species based on the number of sites sampled within the basin.

European carp, *Cyprinus carpio*

Cyprinus carpio is a widespread species, occupying 24 river basins based on the records in the VBA. This species was detected in 25 river basins with eDNA sampling, 24 of which were observed previously according to VBA. eDNA sampling therefore detected *Cyprinus carpio* in 1 river basin for which there were no corresponding VBA records (Otways Coast); this could represent a false positive site detection and warrants further investigation. The only basins in which *Cyprinus carpio* was not detected with eDNA sampling are East Gippsland, Millicent Coast, Portland Coast and Snowy River.

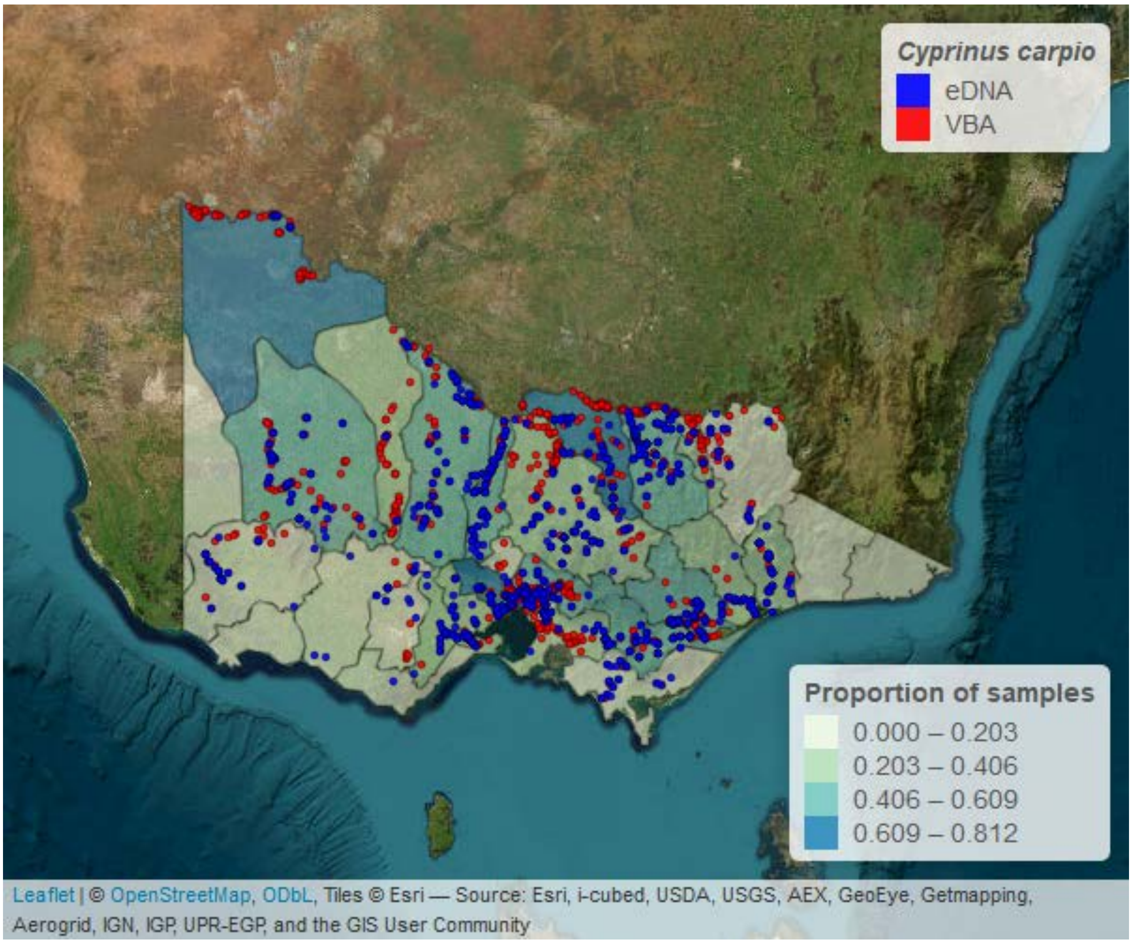

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Figure 37. Detections of *C. carpio* with eDNA (blue dots) and those recorded in the VBA (red dots) for the last 20 years (2003-2023). Basins are colour coded for the proportion of eDNA detections positive for the species based on the number of sites sampled within the basin.

Eastern mosquitofish, *Gambusia holbrooki*

Gambusia holbrooki is a widespread species, occupying 27 river basins based on the records in the VBA. This species was detected in 25 river basins with eDNA sampling, 24 of which were observed previously according to VBA. eDNA sampling therefore detected *Gambusia holbrooki* in 1 river basin for which there were no corresponding VBA records (Tambo River); this may represent a false positive site detection and warrants further investigation. The only basins in which *Gambusia holbrooki* was not detected with eDNA sampling are East Gippsland, Millicent Coast, Portland Coast and Snowy River.

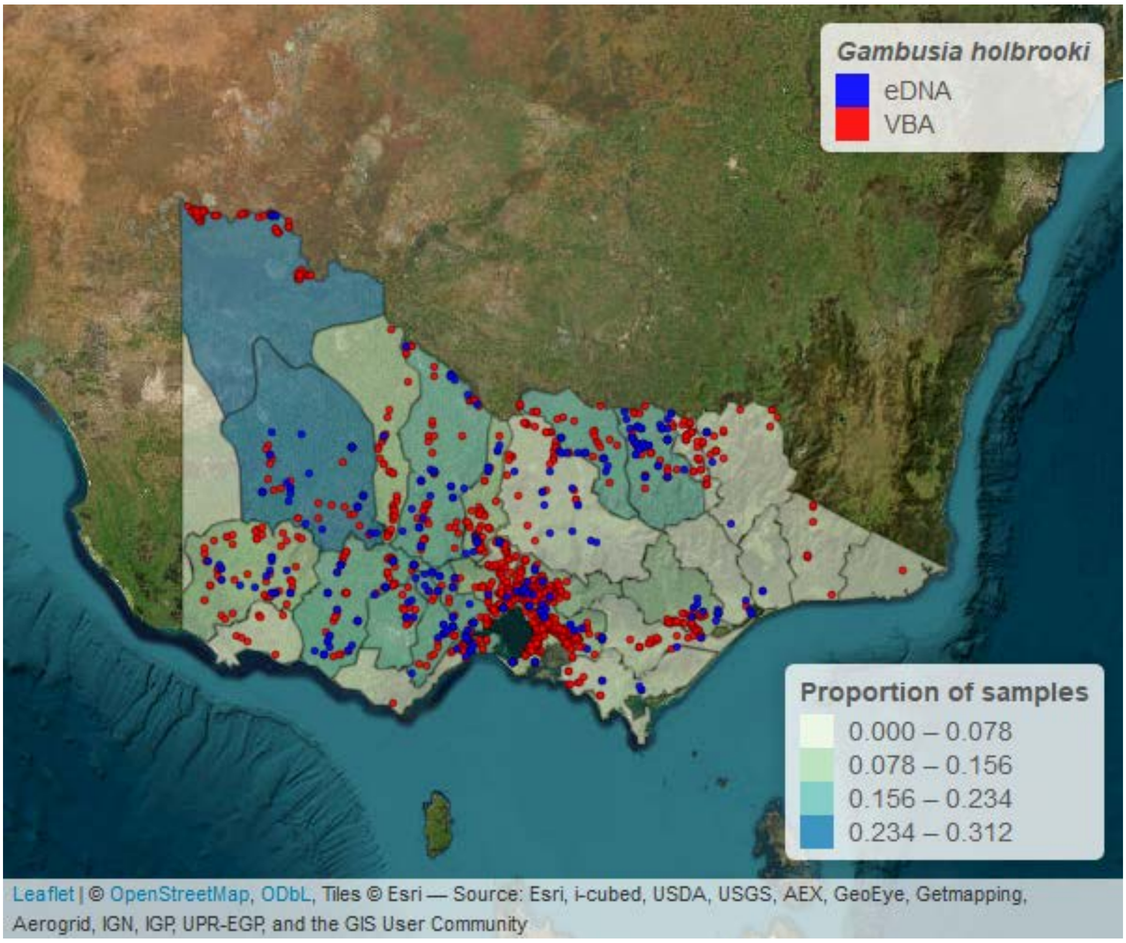

Figure 38. Detections of *Gambusia holbrooki* with eDNA (blue dots) and those recorded in the VBA (red dots) for the last 20 years (2003-2023). Basins are colour coded for the proportion of eDNA detections positive for the species based on the number of sites sampled within the basin.

Oriental weatherloach, *Misgurnus anguillicaudatus*

Misgurnus anguillicaudatus occupies 11 river basins based on the records in the VBA. This species was detected in 12 river basins with eDNA sampling, 9 of which were observed previously according to VBA. eDNA sampling therefore detected *Misgurnus anguillicaudatus* in 3 river basins for which there were no corresponding VBA records (Upper Murray River, Mitchell River, Werribee River). These new detections warrant further investigation to determine whether this invasive species has spread to these basins.

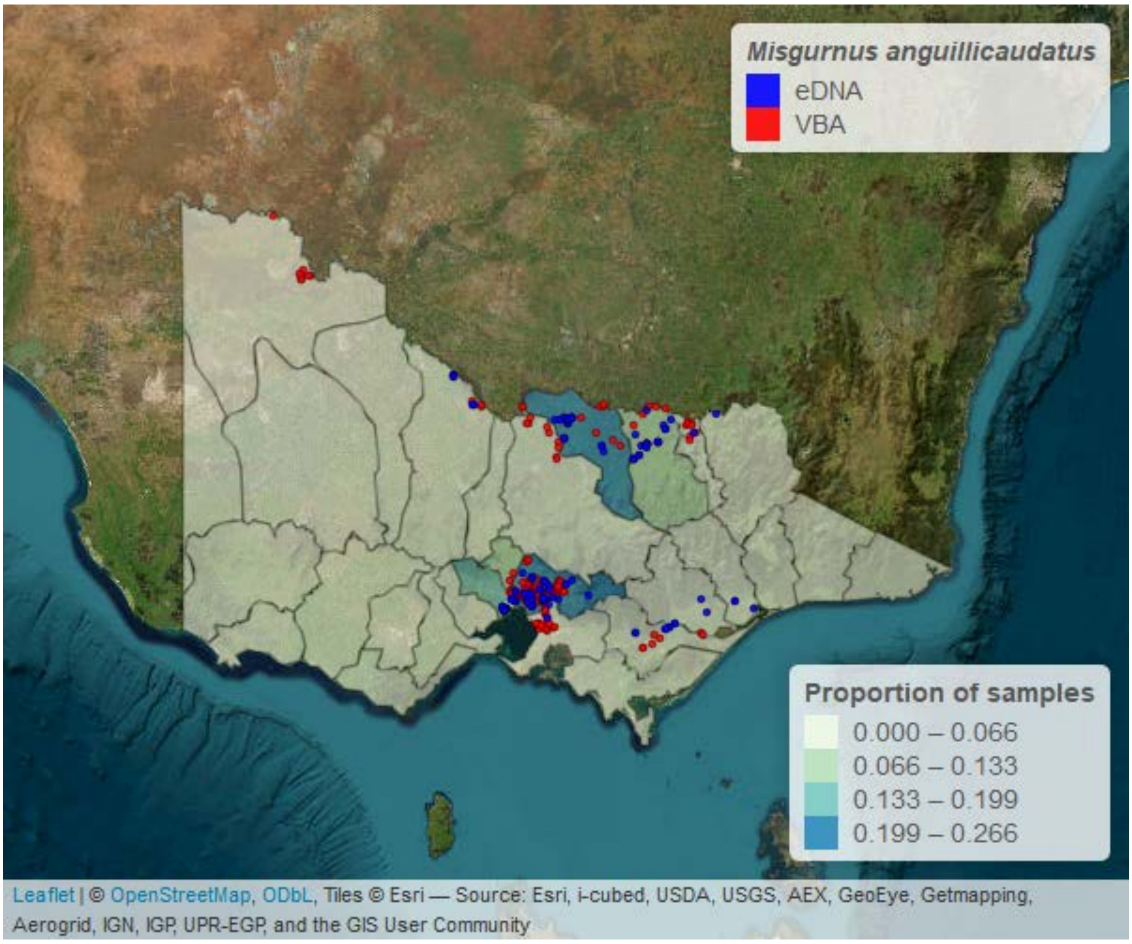

Figure 39. Detections of *M. anguillicaudatus* with eDNA (blue dots) and those recorded in the VBA (red dots) for the last 20 years (2003-2023). Basins are colour coded for the proportion of eDNA detections positive for the species based on the number of sites sampled within the basin.

Rainbow trout, *Oncorhynchus mykiss*

Oncorhynchus mykiss occupies 17 river basins based on the records in the VBA. This species was detected in 17 river basins with eDNA sampling, 12 of which were observed previously according to VBA. eDNA sampling therefore detected *Oncorhynchus mykiss* in 5 river basins for which there were no corresponding VBA records. Some of these are single eDNA site detections within a basin (e.g. South Gippsland) and could represent false positive site detections, although historical records (>20 years old) are known from all five basins.

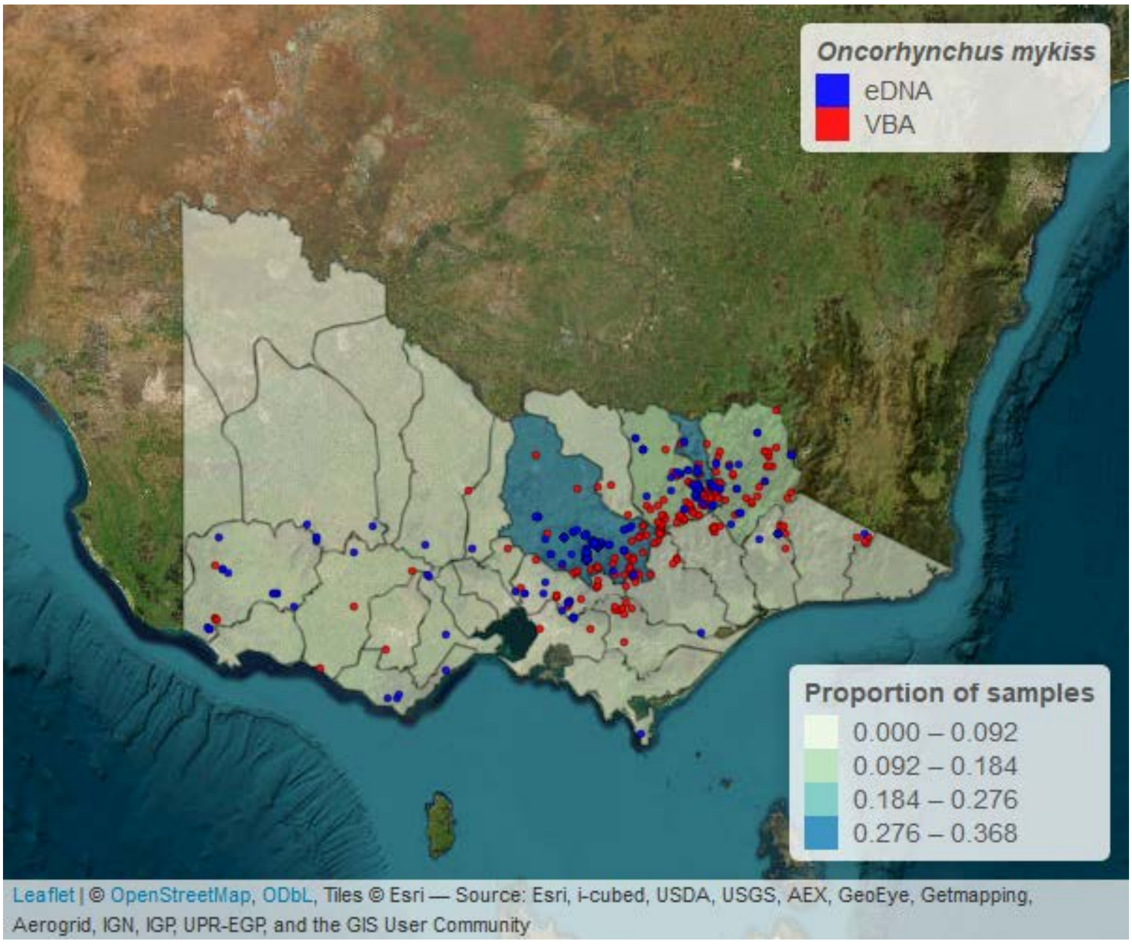

Figure 40. Detections of *O. mykiss* with eDNA (blue dots) and those recorded in the VBA (red dots) for the last 20 years (2003-2023). Basins are colour coded for the proportion of eDNA detections positive for the species based on the number of sites sampled within the basin.

Redfin (European perch), *Perca fluviatilis*

Perca fluviatilis is a widespread invasive species, occupying 26 river basins based on the records in the VBA. This species was detected in 26 river basins with eDNA sampling, 24 of which were observed previously according to VBA. eDNA sampling therefore detected *Perca fluviatilis* in 2 river basins for which there were no corresponding VBA records (Mitchell River and Millicent Coast), although both basins contain records from >20 years ago. The only basins in which *Perca fluviatilis* was not detected with eDNA sampling are Avoca River, East Gippsland and Tambo River.

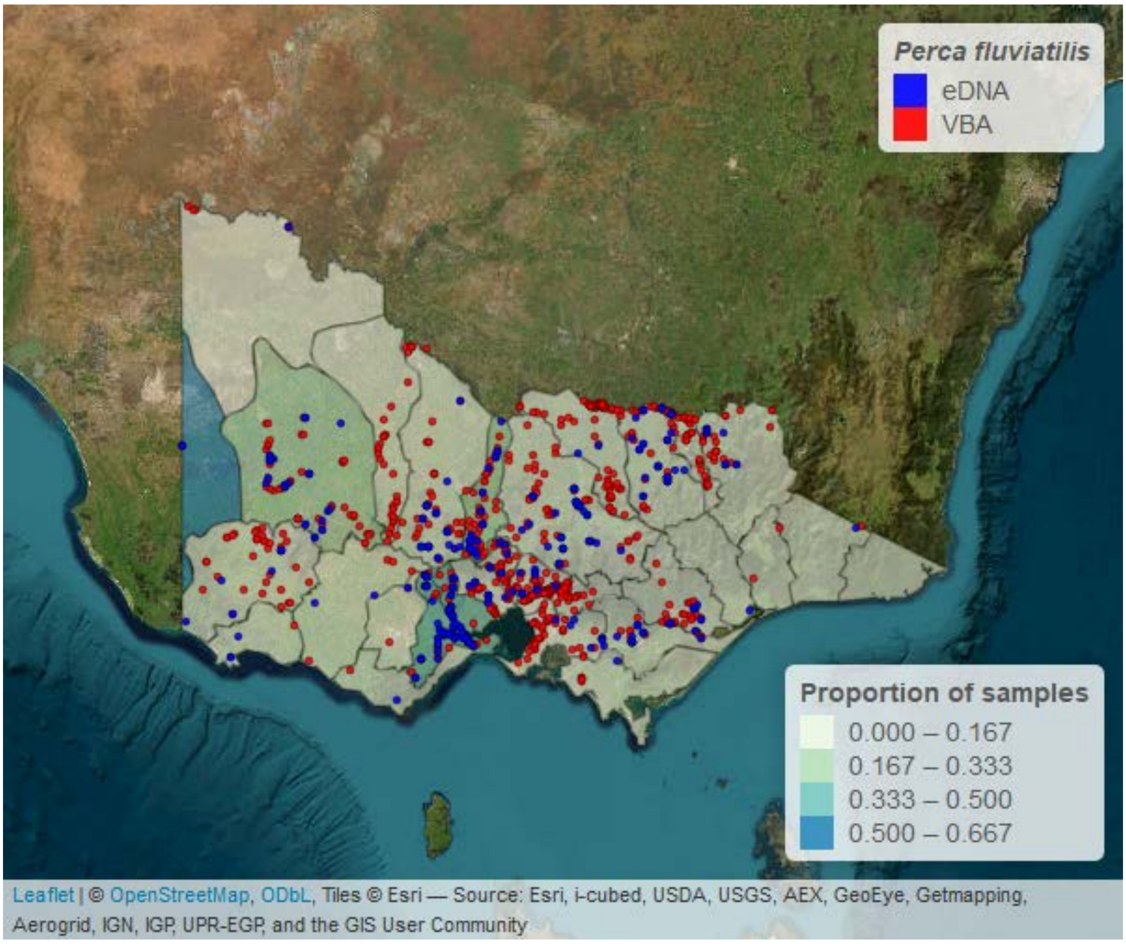

Figure 41. Detections of *P. fluviatilis* with eDNA (blue dots) and those recorded in the VBA (red dots) for the last 20 years (2003-2023). Basins are colour coded for the proportion of eDNA detections positive for the species based on the number of sites sampled within the basin.

Common roach, *Rutilus rutilus*

Rutilus rutilus occupies 9 river basins based on the records in the VBA. This species was detected in 11 river basins with eDNA sampling, 7 of which were observed previously according to VBA. eDNA sampling therefore detected *Rutilus rutilus* in 4 river basins (Ovens River, Thomson River, Latrobe River, Barwon River) for which there were no corresponding VBA records. These eDNA detections warrant further investigation to determine whether they are false positive site detections or that the species has spread to these basins.

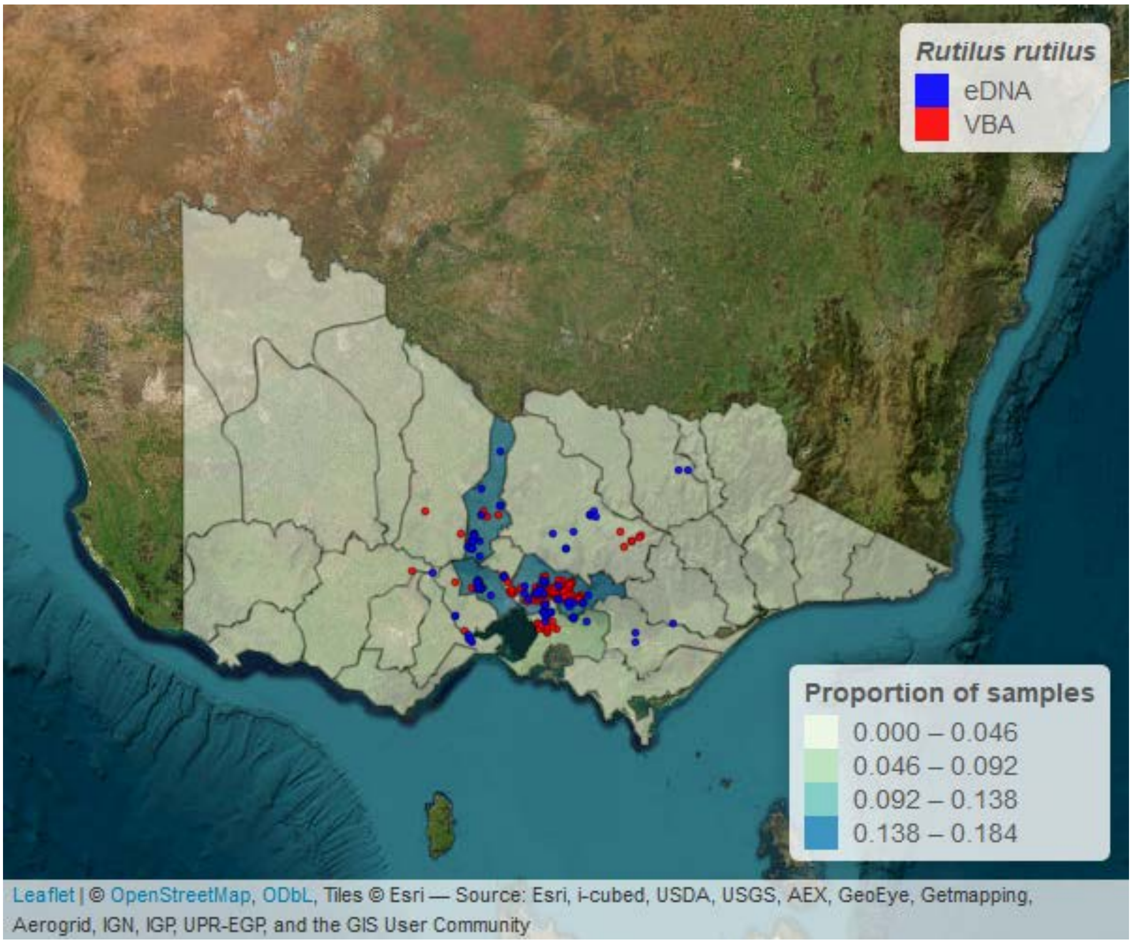

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Figure 42. Detections of *R. rutilus* with eDNA (blue dots) and those recorded in the VBA (red dots) for the last 20 years (2003-2023). Basins are colour coded for the proportion of eDNA detections positive for the species based on the number of sites sampled within the basin.

Brown trout, *Salmo trutta*

Salmo trutta is a widespread introduced recreational fishing species, occupying 25 river basins based on the records in the VBA. This species was detected in 26 river basins with eDNA sampling, 24 of which were observed previously according to VBA. eDNA sampling therefore detected *Salmo trutta* in 2 river basins for which there were no corresponding VBA records (Glenelg River, Avoca River) over the last 20 years (although they have been recorded in both basins >20 years ago in the VBA). The only basins in which *Salmo trutta* was not detected with eDNA sampling are Lake Corangamite, Mallee and Millicent Coast.

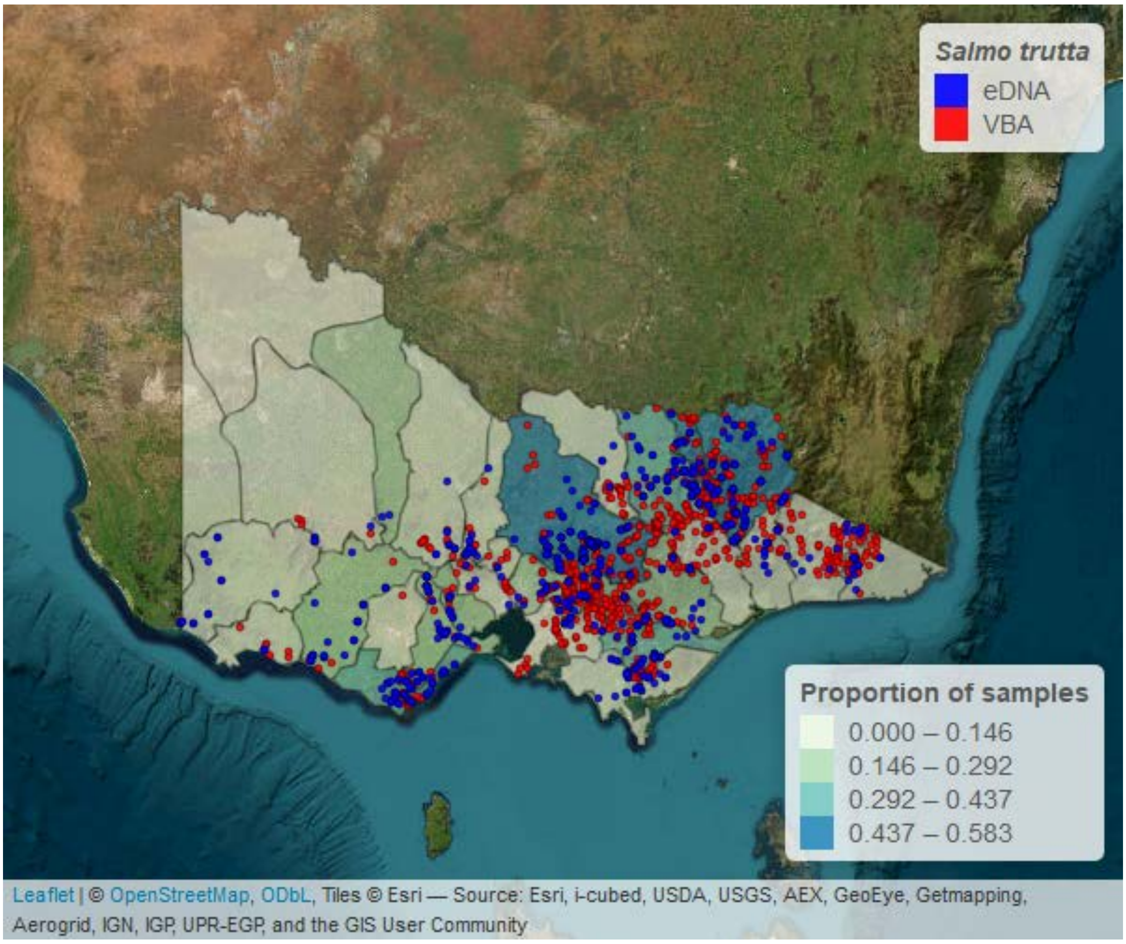

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Figure 43. Detections of *S. trutta* with eDNA (blue dots) and those recorded in the VBA (red dots) for the last 20 years (2003-2023). Basins are colour coded for the proportion of eDNA detections positive for the species based on the number of sites sampled within the basin.

Tench, *Tinca tinca*

Tinca tinca is a widespread invasive species, occupying 20 river basins based on the records in the VBA. This species was detected in 17 river basins with eDNA sampling, 15 of which were observed previously according to VBA. eDNA sampling therefore detected *Tinca tinca* in 2 river basins for which there were no corresponding VBA records (East Gippsland, Mitchell River) in the last 20 years. These may be potential false positive site detections, as both were only single eDNA detections in each basin.

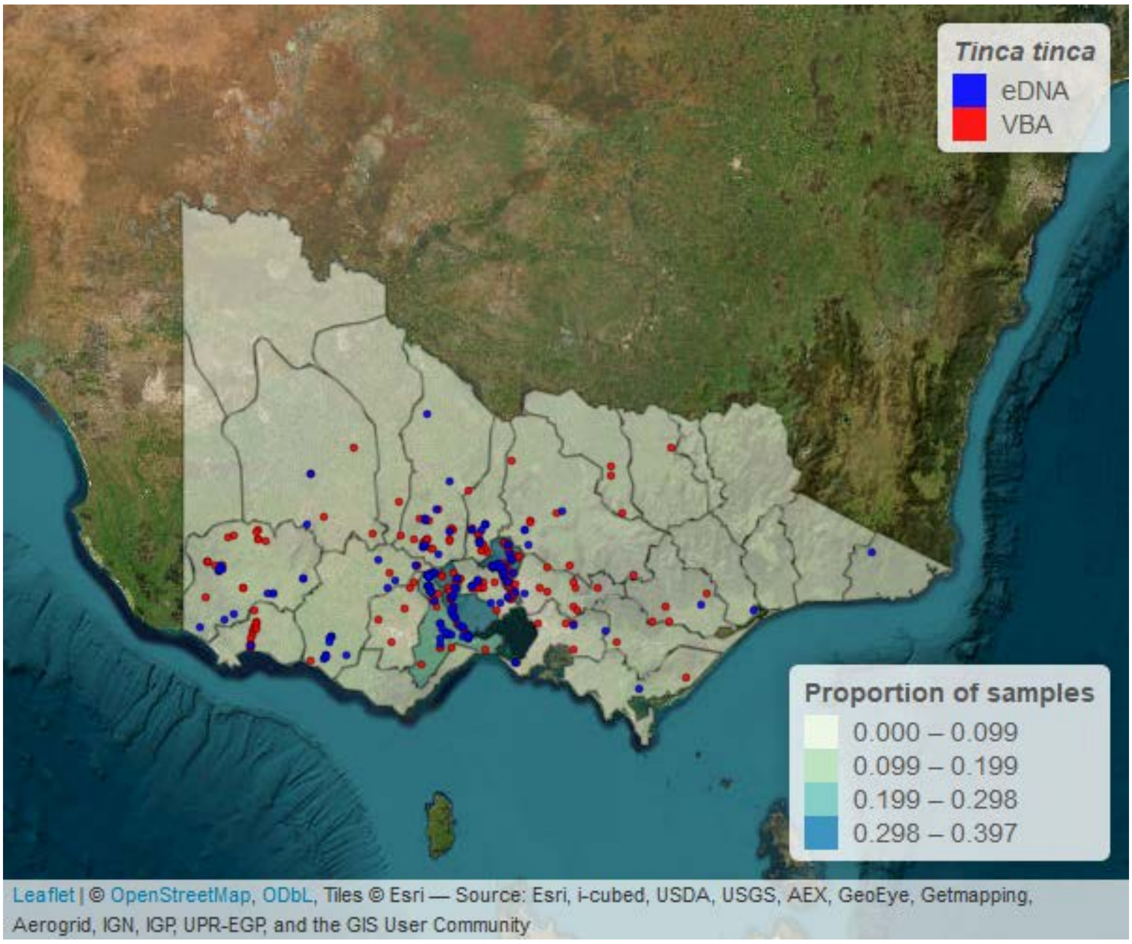

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Figure 44. Detections of *T. tinca* with eDNA (blue dots) and those recorded in the VBA (red dots) for the last 20 years (2003-2023). Basins are colour coded for the proportion of eDNA detections positive for the species based on the number of sites sampled within the basin.

Other threatened vertebrate species detected

There were a large number of terrestrial or semi-aquatic vertebrate species detected through the eDNA samples. These are largely non-target species detections, as the water eDNA sampling technique generally targets aquatic species. However, the eDNA detection data from these samples highlights the potential use of eDNA from water samples to also detect terrestrial and semi-aquatic species. For instance, a number of terrestrial species were commonly detected, with the detections matching the distributions for these species on ALA e.g., *Rusa unicolor* (Sambar deer), *Trichosurus vulpecula* (brushtail possum). All terrestrial and semi-aquatic eDNA detections can be viewed in the Shiny app (https://envirodna.shinyapps.io/GVWS_release/).

There were also sporadic detections of several threatened terrestrial / semi-aquatic species, some of which appear in new locations, or in locations where there have been no records over the last 20 years in the ALA. We highlight all threatened species detections for these taxa below. We don't include platypuses, as these have previously been reported on using qPCR (see Griffiths et al. 2022).

Frogs and turtles

There were 16 frog taxa detected from the eDNA surveys, with detections of three FFG listed frog species. Both *Litoria aurea* (Green and Golden Bell Frog) and *Litoria raniformis* (Growling Grass Frog) were detected in areas where they have previously been recorded on ALA). There were also a number of detections stuck at the genus level (*Litoria*) in areas where these two species are known to occur that could represent further detections of these species (e.g., East Gippsland, Snowy River and Tambo River basins). There were four site detections of Bibron's toadlet (*Pseudophryne bibronii*) in four separate basins of eastern Victoria, all close to previous detections of this species in ALA, but given the rapid decline in this species, these detections are significant. There were also four turtle taxa detected, with detections of two threatened turtle species, *Chelodina expansa* (Broad-shelled turtle) and *Emydura macquarii* (Murray River turtle). The detections of these five threatened species are shown in Figure 45.

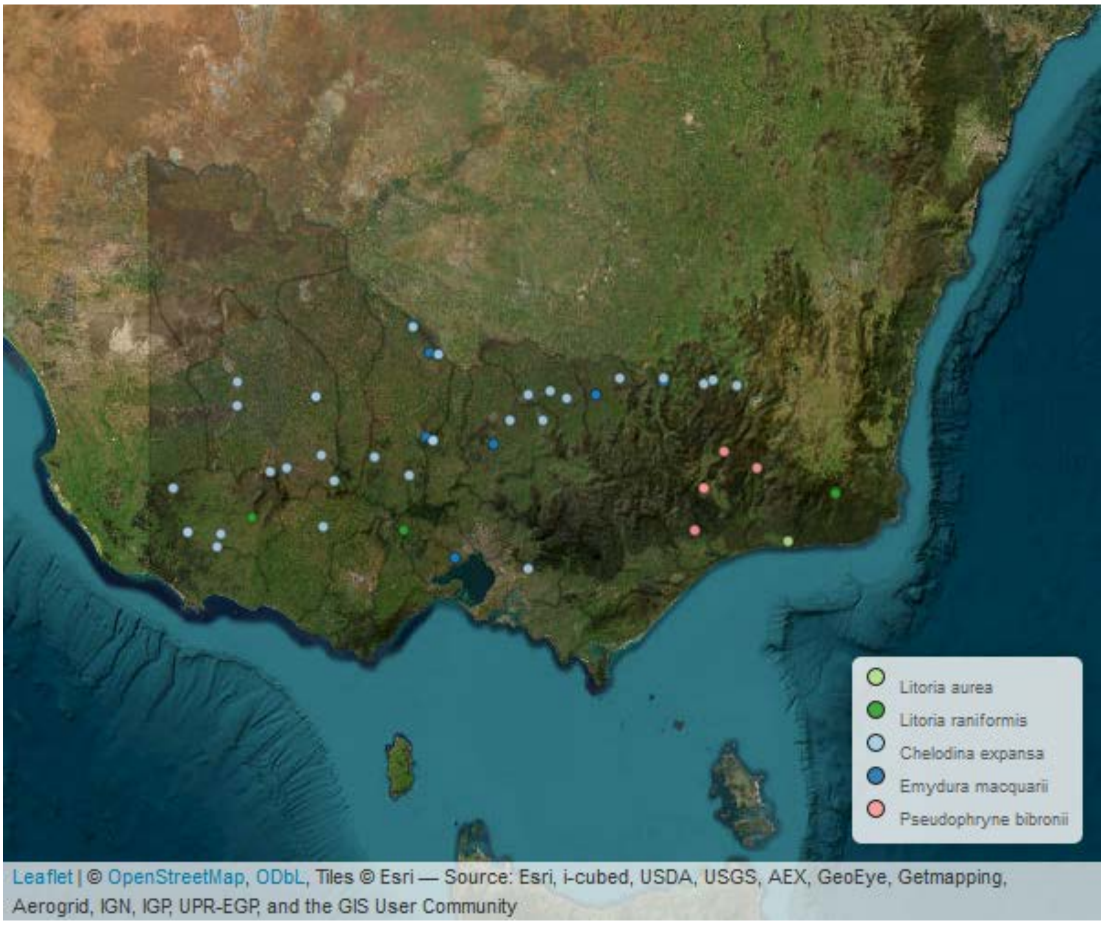

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Figure 45. Detections of three threatened frog species (*Litoria aurea*, *Litoria raniformis*, *Pseudophryne bibronii*) and two turtles (*Chelodina expansa*, *Emydura macquarii*) from eDNA samples.

Mammals

A total of 41 mammal taxa were detected from the eDNA samples, with detections of six FFG listed mammal species. The Southern greater glider (*Petauroides volans*) was detected at 9 sites, representing four basins. These detections are all known locations of Southern greater gliders in ALA. The southern brown bandicoot (*Isoodon obesulus*), was detected at two sites (both known locations in ALA), while the yellow bellied glider (*Petaurus australis*), long-nosed potoroo (*Potorous tridactylus*) and grey-headed flying-fox (*Pteropus poliocephalus*) were all detected in single sites that are in the area of previous records on ALA. The broad-toothed rat was detected at 7 sites, some of which appear new locations (e.g. near Mount Reynard in the Thomson River Basin) or locations where they have not been recorded for a long period of time (e.g. Bunyip and Otways). These detections warrant further investigations.

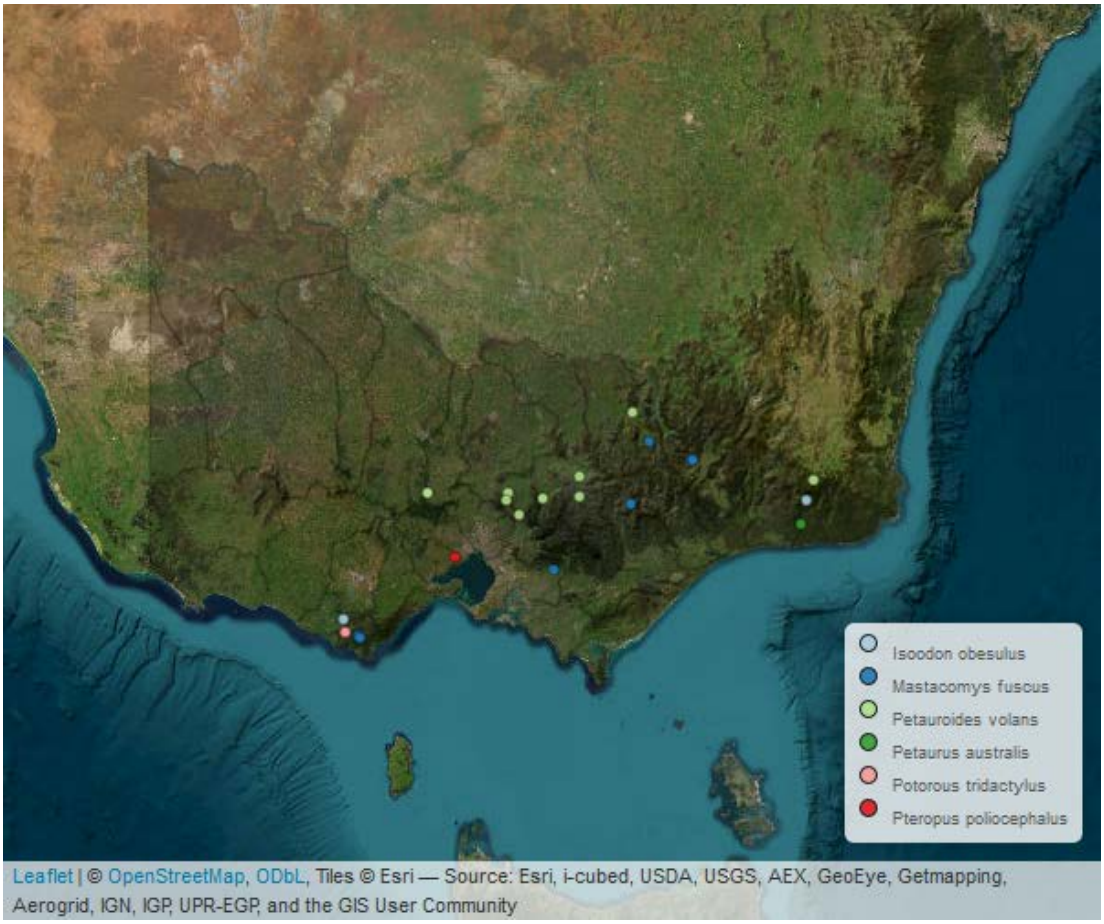

Figure 46. Detections of six threatened mammal species (*Isoodon obesulus, Mastacomys fuscus, Petauroides volans, Petaurus australis, Potorous tridactylus, Pteropus poliocephalus*) from eDNA samples.

Birds

While we detected 77 bird taxa, there was only one FFG listed species, Lewin's rail (*Lewinia pectoralis*), detected from the eDNA samples at 13 sites. All sites were within the known range of the species on ALA. Generally, there was relatively poor resolution of many bird taxa with the Vertebrate amplicon, with many detections not being able to be resolved further than Genus or Family levels.

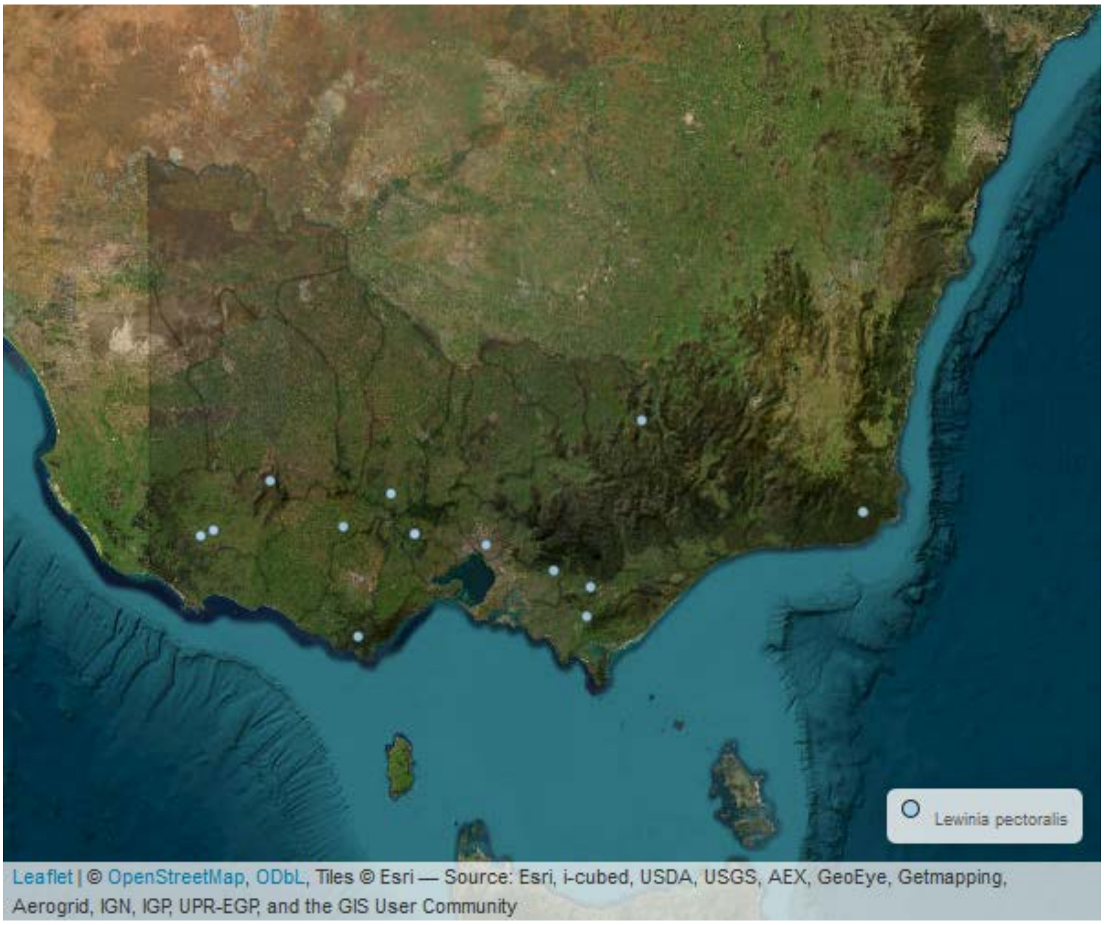

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Figure 47. Detections of a threatened bird species (*Lewinia pectoralis)* from eDNA samples.

Discussion

Using eDNA techniques and citizen scientists, we obtained vertebrate biodiversity detection data for 1850 unique sites across Victoria's river network in the largest spatial eDNA survey undertaken. We detected 224 vertebrate taxa, with 77 fish (including cartilaginous and jawless fish), 41 mammals, 77 birds, 16 amphibians, and 13 reptiles. This included 30 FFG listed threatened species, including 16 fish, 3 frogs, 1 bird, 2 reptiles and 8 mammals. There are likely to be numerous more threatened fish taxa detections that are stuck at genus level because we were unable to differentiate between some species; these include detections of *Nannoperca* sp. (possible *N. obscura*), *Hypseleotris* spp. (possible *H. compressa*), and numerous species within the *Galaxias olidus* species complex (e.g. *G. fuscus*, *G. aequipinnis*, *G. mungadhan*, *G. mcdowalli*, *G. longifundus* etc).

The eDNA survey program was designed to provide aquatic vertebrate biodiversity data across Victoria's river network, utilizing a Generalised Random Tessellation Stratified design (GRTS). This design was chosen to provide a balanced unbiased design that adequately reflects the dominant environmental gradients involved in determining species distributions and that the survey sites covered stream reaches with existing local data (Shackleton et al. 2021). The sites were also somewhat ground-truthed (where possible) by local Catchment Management Authorities, to confirm feasibility of access and increase the likelihood of flowing water present at sites. Citizen scientists then undertook the sampling over 3-4 months during spring and early summer 2021 (some delays were experienced due to Covid-19 lockdowns in Victoria, which also impacted samples being returned to the laboratory for analysis). Unfortunately, some samples yielded low (or no) sequence reads, which could be due to the amount of water filtered (sample volumes varied widely from 7 mL to 3430 mL), the time delays between sampling and arriving in the laboratory (some samples took up to 3 weeks before arriving at EnviroDNA due to postage issues) or simply that there was low DNA amounts in the water (e.g. because of very recent rainfall).

However, for a single one-off survey, the eDNA detections were generally comparable to existing data held in the VBA for aquatic species from the last 20 years. This highlights the potential of eDNA to provide rapid data over large spatial scales on species distributions. The method was highly effective for determining the distribution of platypuses from the same samples using qPCR (Griffith et al. 2022), and here we have been able to provide extensive data for over 40 aquatic freshwater fish species using eDNA metabarcoding. There was generally good agreement between historic data and the eDNA detections, although there are likely to be some false positive and false negative site detections for some taxa. False negatives (no eDNA detection when a species occupies a site) can be due to many different reasons including how the sample was taken (e.g., volume of water, where the sample was taken within the river), environmental conditions at the time of sampling (e.g., recent rainfall can dilute DNA), and detectability of the species (e.g., abundance at the site, DNA concentration excreted by the species, DNA degradation).

We have attempted to control for false positives at the sample level in the laboratory, but recognize that there are likely to be some false positives that are due to index switching, overlapping ASVs between taxa or a lack of reference sequences enabling correct identification. We outline in Appendix 2 some method improvements that could be incorporated into future eDNA surveys that will reduce the number of false positive detections at the sample level within the laboratory. The key improvements will include using UDIs (which prevents index switching) and multiple amplicons targeting individuals groups (which will increase detections and confidence of detections).

Similarly, there are possible false positives at the site level that could be due to movement of DNA downstream or by animals, and sample / metadata mix-ups due to how data was recorded by citizen scientists. Follow-up surveys using eDNA or other sampling methods may be needed to differentiate for important detections (e.g., a species detected in a new waterway / basin). Similarly, detections at the genus level that may also be important (e.g., *G. olidus* species complex in a new waterway in Gippsland) could be followed up with traditional surveys, or new reference material collected to help differentiate detections (see Appendix 2). In Appendix 3 we have highlighted some freshwater native species detections that are worth following up either with additional eDNA based surveys or traditional surveys. These are detections in potentially new basins for the species.

The vast amount of eDNA detection data for aquatic taxa gathered during this project highlights the opportunities that eDNA methods present for large spatial surveys in aquatic environments. By coupling this program with citizen science sampling, costs were vastly reduced and enabled a relatively efficient sampling program to be undertaken in a short period of time. There was also a large number of detections of terrestrial and semi-aquatic taxa from these waterway samples, and while these were generally non-target taxa, this demonstrates additional benefits of eDNA sampling for land and waterway managers. Baselining and monitoring taxa distributions in real-time are likely to be necessary, particularly when information is needed to respond to environmental catastrophes (McColl-Gausden et al. 2023). This project has highlighted the potential for eDNA methods to be used for this purpose.

Acknowledgments

The 2021 Great Australian Platypus Search was the result of a strong collaboration by many organisations. However, the project would not have been possible without the contribution of local citizen scientists, and the project team would like to thank them for their assistance. We would also like to thank the following key project partners and donors:

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Victorian Department of Environment, Land, Water and Planning
Parks Victoria
Outback Academy Australia
The Ross Trust
Waterwatch Victoria
Capricorn Foundation
2040
Environment Education Victoria
Planet Warrior Education
Fouress Foundation
PMF Foundation
Carbon Landscapes
Latrobe University

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Appendix 1. Taxa detected from eDNA site surveys

Kingdom	Phylum	Class	Order	Family	Genus	Species	N site detections
Metazoa	Chordata	Actinopteri	Anguilliformes	Anguillidae	Anguilla	Anguilla australis	607
Metazoa	Chordata	Actinopteri	Anguilliformes	Anguillidae	Anguilla	Anguilla reinhardtii	142
Metazoa	Chordata	Actinopteri	Atheriniformes	Atherinidae	Atherinosoma	Atherinosoma microstoma	6
Metazoa	Chordata	Actinopteri	Atheriniformes	Atherinidae	Craterocephalus	Craterocephalus fulvus	7
Metazoa	Chordata	Actinopteri	Atheriniformes	Melanotaeniidae	Melanotaenia	Melanotaenia fluviatilis	9
Metazoa	Chordata	Actinopteri	Carangiformes	Carangidae	Decapterus	Decapterus macarellus	1
Metazoa	Chordata	Actinopteri	Centrarchiformes	Percichthyidae	Gadopsis	Gadopsis bispinosus	116
Metazoa	Chordata	Actinopteri	Centrarchiformes	Percichthyidae	Gadopsis	Gadopsis marmoratus	201
Metazoa	Chordata	Actinopteri	Centrarchiformes	Percichthyidae	Gadopsis	Gadopsis sp.	81
Metazoa	Chordata	Actinopteri	Centrarchiformes	Percichthyidae	Maccullochella	Maccullochella macquariensis	14
Metazoa	Chordata	Actinopteri	Centrarchiformes	Percichthyidae	Maccullochella	Maccullochella peelii	69
Metazoa	Chordata	Actinopteri	Centrarchiformes	Percichthyidae	Macquaria	Macquaria ambigua	50
Metazoa	Chordata	Actinopteri	Centrarchiformes	Percichthyidae	Macquaria	Macquaria australasica	25
Metazoa	Chordata	Actinopteri	Centrarchiformes	Percichthyidae	Macquaria	Macquaria sp.	2
Metazoa	Chordata	Actinopteri	Centrarchiformes	Percichthyidae	Nannoperca	Nannoperca sp.	358
Metazoa	Chordata	Actinopteri	Centrarchiformes	Percichthyidae	Nannoperca	Nannoperca australis	382
Metazoa	Chordata	Actinopteri	Centrarchiformes	Percichthyidae	Nannoperca	Nannoperca variegata	16
Metazoa	Chordata	Actinopteri	Centrarchiformes	Terapontidae	Bidyanus	Bidyanus bidyanus	1
Metazoa	Chordata	Actinopteri	Centrarchiformes	Terapontidae	Terapontidae sp.		6
Metazoa	Chordata	Actinopteri	Clupeiformes	Clupeidae	Hyperlophus	Hyperlophus vittatus	2
Metazoa	Chordata	Actinopteri	Clupeiformes	Clupeidae	Nematalosa	Nematalosa erebi	8
Metazoa	Chordata	Actinopteri	Clupeiformes	Clupeidae	Sardinops	Sardinops sp.	2
Metazoa	Chordata	Actinopteri	Cypriniformes	Cobitidae	Misgurnus	Misgurnus anguillicaudatus	70
Metazoa	Chordata	Actinopteri	Cypriniformes	Cyprinidae	Carassius	Carassius auratus	238

Kingdom	Phylum	Class	Order	Family	Genus	Species	N site detections
Metazoa	Chordata	Actinopteri	Cypriniformes	Cyprinidae	Cyprinus	Cyprinus carpio	511
Metazoa	Chordata	Actinopteri	Cypriniformes	Leuciscidae	Rutilus	Rutilus rutilus	63
Metazoa	Chordata	Actinopteri	Cypriniformes	Tincidae	Tinca	Tinca tinca	108
Metazoa	Chordata	Actinopteri	Cyprinodontiformes	Poeciliidae	Gambusia	Gambusia holbrooki	210
Metazoa	Chordata	Actinopteri	Galaxiiformes	Galaxiidae	Galaxias	Galaxias brevipinnis	134
Metazoa	Chordata	Actinopteri	Galaxiiformes	Galaxiidae	Galaxias	Galaxias maculatus	351
Metazoa	Chordata	Actinopteri	Galaxiiformes	Galaxiidae	Galaxias	Galaxias olidus complex	366
Metazoa	Chordata	Actinopteri	Galaxiiformes	Galaxiidae	Galaxias	Galaxias truttaceus	37
Metazoa	Chordata	Actinopteri	Galaxiiformes	Galaxiidae	Galaxias	Galaxias rostratus	1
Metazoa	Chordata	Actinopteri	Galaxiiformes	Galaxiidae	Galaxias	Galaxias sp.	1
Metazoa	Chordata	Actinopteri	Galaxiiformes	Galaxiidae	Galaxiella	Galaxiella pusilla	10
Metazoa	Chordata	Actinopteri	Galaxiiformes	Galaxiidae	Galaxiella	Galaxiella toourtkoourt	31
Metazoa	Chordata	Actinopteri	Galaxiiformes	Galaxiidae	Neochanna	Neochanna cleaveri	5
Metazoa	Chordata	Actinopteri	Gobiiformes	Eleotridae	Gobiomorphus	Gobiomorphus coxii	3
Metazoa	Chordata	Actinopteri	Gobiiformes	Eleotridae	Hypseleotris	Hypseleotris spp.	110
Metazoa	Chordata	Actinopteri	Gobiiformes	Eleotridae	Philypnodon	Philypnodon grandiceps	349
Metazoa	Chordata	Actinopteri	Gobiiformes	Eleotridae	Philypnodon	Philypnodon macrostomus	50
Metazoa	Chordata	Actinopteri	Gobiiformes	Gobiidae	Acanthogobius	Acanthogobius flavimanus	1
Metazoa	Chordata	Actinopteri	Gobiiformes	Gobiidae	Afurcagobius	Afurcagobius tamarensis	15
Metazoa	Chordata	Actinopteri	Gobiiformes	Gobiidae	Arenigobius	Arenigobius frenatus	9
Metazoa	Chordata	Actinopteri	Gobiiformes	Gobiidae	Arenigobius	Arenigobius bifrenatus	6
Metazoa	Chordata	Actinopteri	Gobiiformes	Gobiidae	Gobiopterus	Gobiopterus semivestitus	3
Metazoa	Chordata	Actinopteri	Gobiiformes	Gobiidae	Mugilogobius	Mugilogobius platynotus	1
Metazoa	Chordata	Actinopteri	Gobiiformes	Gobiidae	Pseudogobius	Pseudogobius olorum	26
Metazoa	Chordata	Actinopteri	Gobiiformes	Gobiidae	Redigobius	Redigobius macrostoma	2
Metazoa	Chordata	Actinopteri	Gobiiformes	Gobiidae	Tasmanogobius	Tasmanogobius lasti	7

Kingdom	Phylum	Class	Order	Family	Genus	Species	N site detections
Metazoa	Chordata	Actinopteri	Mugiliformes	Mugilidae	Aldrichetta	Aldrichetta forsteri	7
Metazoa	Chordata	Actinopteri	Mugiliformes	Mugilidae	Gracilimugil	Gracilimugil argenteus	2
Metazoa	Chordata	Actinopteri	Mugiliformes	Mugilidae	Mugil	Mugil cephalus	6
Metazoa	Chordata	Actinopteri	Osmeriformes	Retropinnidae	Prototroctes	Prototroctes maraena	6
Metazoa	Chordata	Actinopteri	Osmeriformes	Retropinnidae	Retropinna	Retropinna semoni	489
Metazoa	Chordata	Actinopteri	Perciformes	Percichthyidae	Percalates	Macquaria colonorum	21
Metazoa	Chordata	Actinopteri	Perciformes	Percichthyidae	Percalates	Macquaria novemaculeata	19
Metazoa	Chordata	Actinopteri	Perciformes	Percidae	Perca	Perca fluviatilis	204
Metazoa	Chordata	Actinopteri	Perciformes	Pseudaphritidae	Pseudaphritis	Pseudaphritis urvillii	174
Metazoa	Chordata	Actinopteri	Pleuronectiformes	Rhombosoleidae	Rhombosolea	Rhombosolea tapirina	2
Metazoa	Chordata	Actinopteri	Salmoniformes	Salmonidae	Oncorhynchus	Oncorhynchus mykiss	118
Metazoa	Chordata	Actinopteri	Salmoniformes	Salmonidae	Oncorhynchus	Oncorhynchus tshawytscha	5
Metazoa	Chordata	Actinopteri	Salmoniformes	Salmonidae	Salmo	Salmo salar	12
Metazoa	Chordata	Actinopteri	Salmoniformes	Salmonidae	Salmo	Salmo trutta	387
Metazoa	Chordata	Actinopteri	Salmoniformes	Salmonidae	Salvelinus	Salvelinus fontinalis	25
Metazoa	Chordata	Actinopteri	Scombriformes	Arripidae	Arripis	Arripis trutta	3
Metazoa	Chordata	Actinopteri	Siluriformes	Plotosidae	Tandanus	Tandanus tandanus	9
Metazoa	Chordata	Actinopteri	Spariformes	Sillaginidae	Sillaginodes	Sillaginodes punctatus	1
Metazoa	Chordata	Actinopteri	Spariformes	Sparidae	Acanthopagrus	Acanthopagrus butcheri	22
Metazoa	Chordata	Actinopteri	Spariformes	Sparidae	Pagrus	Pagrus sp.	3
Metazoa	Chordata	Actinopteri	Tetraodontiformes	Tetraodontidae	Tetractenos	Tetractenos glaber	2
Metazoa	Chordata	Amphibia	Anura	Hylidae	Litoria	Litoria aurea	1
Metazoa	Chordata	Amphibia	Anura	Hylidae	Litoria	Litoria ewingii	59
Metazoa	Chordata	Amphibia	Anura	Hylidae	Litoria	Litoria lesueuri	70
Metazoa	Chordata	Amphibia	Anura	Hylidae	Litoria	Litoria peronii	6
Metazoa	Chordata	Amphibia	Anura	Hylidae	Litoria	Litoria raniformis	4

Kingdom	Phylum	Class	Order	Family	Genus	Species	N site detections
Metazoa	Chordata	Amphibia	Anura	Hylidae	Litoria	Litoria sp.	13
Metazoa	Chordata	Amphibia	Anura	Limnodynastidae	Limnodynastes	Limnodynastes dumerilii	192
Metazoa	Chordata	Amphibia	Anura	Limnodynastidae	Limnodynastes	Limnodynastes peronii	65
Metazoa	Chordata	Amphibia	Anura	Limnodynastidae	Limnodynastes	Limnodynastes tasmaniensis	68
Metazoa	Chordata	Amphibia	Anura	Limnodynastidae	Neobatrachus	Neobatrachus sudellae	18
Metazoa	Chordata	Amphibia	Anura	Myobatrachidae	Crinia	Crinia parinsignifera	14
Metazoa	Chordata	Amphibia	Anura	Myobatrachidae	Crinia	Crinia signifera	339
Metazoa	Chordata	Amphibia	Anura	Myobatrachidae	Crinia	Crinia sp.	5
Metazoa	Chordata	Amphibia	Anura	Myobatrachidae	Geocrinia	Geocrinia victoriana	34
Metazoa	Chordata	Amphibia	Anura	Myobatrachidae	Paracrinia	Paracrinia haswelli	4
Metazoa	Chordata	Amphibia	Anura	Myobatrachidae	Pseudophryne	Pseudophryne bibronii	4
Metazoa	Chordata	Aves	Accipitriformes	Accipitridae	Aquila	Aquila audax	1
Metazoa	Chordata	Aves	Accipitriformes	Accipitridae	Circus	Circus assimilis	1
Metazoa	Chordata	Aves	Anseriformes	Anatidae	Anatidae sp.	Anatidae sp.	688
Metazoa	Chordata	Aves	Anseriformes	Anatidae	Chenonetta	Chenonetta jubata	446
Metazoa	Chordata	Aves	Anseriformes	Anatidae	Tadorna	Tadorna sp.	18
Metazoa	Chordata	Aves	Casuariiformes	Dromaiidae	Dromaius	Dromaius novaehollandiae	11
Metazoa	Chordata	Aves	Charadriiformes	Scolopacidae	Gallinago	Gallinago hardwickii	1
Metazoa	Chordata	Aves	Columbiformes	Columbidae	Columba	Columba livia	14
Metazoa	Chordata	Aves	Columbiformes	Columbidae	Leucosarcia	Leucosarcia melanoleuca	1
Metazoa	Chordata	Aves	Columbiformes	Columbidae	Ocyphaps	Ocyphaps lophotes	9
Metazoa	Chordata	Aves	Columbiformes	Columbidae	Phaps	Phaps chalcoptera	18
Metazoa	Chordata	Aves	Columbiformes	Columbidae	Spilopelia	Spilopelia chinensis	18
Metazoa	Chordata	Aves	Coraciiformes	Alcedinidae	Dacelo	Dacelo novaeguineae	28
Metazoa	Chordata	Aves	Coraciiformes	Alcedinidae	Todiramphus	Todiramphus sanctus	6
Metazoa	Chordata	Aves	Galliformes	Phasianidae	Coturnix	Coturnix pectoralis	1

Kingdom	Phylum	Class	Order	Family	Genus	Species	N site detections
Metazoa	Chordata	Aves	Galliformes	Phasianidae	Gallus	Gallus gallus	318
Metazoa	Chordata	Aves	Galliformes	Phasianidae	Meleagris	Meleagris gallopavo	3
Metazoa	Chordata	Aves	Gruiformes	Rallidae	Fulica	Fulica atra	20
Metazoa	Chordata	Aves	Gruiformes	Rallidae	Gallinula	Gallinula tenebrosa	175
Metazoa	Chordata	Aves	Gruiformes	Rallidae	Lewinia	Lewinia pectoralis	13
Metazoa	Chordata	Aves	Gruiformes	Rallidae	Porphyrio	Porphyrio sp.	77
Metazoa	Chordata	Aves	Gruiformes	Rallidae	Porzana	Porzana tabuensis	20
Metazoa	Chordata	Aves	Passeriformes	Acanthizidae	Acanthiza	Acanthiza nana	1
Metazoa	Chordata	Aves	Passeriformes	Acanthizidae	Acanthiza	Acanthiza pusilla	22
Metazoa	Chordata	Aves	Passeriformes	Acanthizidae	Gerygone	Gerygone mouki	1
Metazoa	Chordata	Aves	Passeriformes	Acanthizidae	Pyrrholaemus	Pyrrholaemus sp.	1
Metazoa	Chordata	Aves	Passeriformes	Acanthizidae	Sericornis	Sericornis frontalis	53
Metazoa	Chordata	Aves	Passeriformes	Alaudidae	Alauda	Alauda arvensis	2
Metazoa	Chordata	Aves	Passeriformes	Artamidae	Gymnorhina	Gymnorhina tibicen	67
Metazoa	Chordata	Aves	Passeriformes	Cisticolidae	Cisticola	Cisticola sp.	18
Metazoa	Chordata	Aves	Passeriformes	Climacteridae	Climacteris	Climacteris picumnus	4
Metazoa	Chordata	Aves	Passeriformes	Climacteridae	Cormobates	Cormobates leucophaea	4
Metazoa	Chordata	Aves	Passeriformes	Corvidae	Corvus	Corvus sp.	82
Metazoa	Chordata	Aves	Passeriformes	Fringillidae	Carduelis	Carduelis carduelis	2
Metazoa	Chordata	Aves	Passeriformes	Hirundinidae	Hirundo	Hirundo sp.	59
Metazoa	Chordata	Aves	Passeriformes	Locustellidae	Poodytes	Poodytes sp.	1
Metazoa	Chordata	Aves	Passeriformes	Meliphagidae	Nesoptilotis	Nesoptilotis leucotis	6
Metazoa	Chordata	Aves	Passeriformes	Meliphagidae	Ptilotula	Ptilotula penicillata	21
Metazoa	Chordata	Aves	Passeriformes	Menuridae	Menura	Menura novaehollandiae	14
Metazoa	Chordata	Aves	Passeriformes	Monarchidae	Grallina	Grallina cyanoleuca	27
Metazoa	Chordata	Aves	Passeriformes	Pardalotidae	Pardalotus	Pardalotus punctatus	6

Kingdom	Phylum	Class	Order	Family	Genus	Species	N site detections
Metazoa	Chordata	Aves	Passeriformes	Pardalotidae	Pardalotus	Pardalotus striatus	5
Metazoa	Chordata	Aves	Passeriformes	Passeridae	Passer	Passer sp.	27
Metazoa	Chordata	Aves	Passeriformes	Petroicidae	Eopsaltria	Eopsaltria australis	9
Metazoa	Chordata	Aves	Passeriformes	Petroicidae	Petroica	Petroica phoenicea	1
Metazoa	Chordata	Aves	Passeriformes	Ptilonorhynchidae	Ptilonorhynchus	Ptilonorhynchus violaceus	4
Metazoa	Chordata	Aves	Passeriformes	Rhipiduridae	Rhipidura	Rhipidura sp.	4
Metazoa	Chordata	Aves	Passeriformes	Sylviidae	Acrocephalus	Acrocephalus sp.	5
Metazoa	Chordata	Aves	Passeriformes	Turdidae	Turdus	Turdus philomelos	8
Metazoa	Chordata	Aves	Passeriformes	Turdidae	Turdus	Turdus sp.	79
Metazoa	Chordata	Aves	Passeriformes	Zosteropidae	Zosterops	Zosterops lateralis	20
Metazoa	Chordata	Aves	Pelecaniformes	Ardeidae	Bubulcus	Bubulcus ibis	10
Metazoa	Chordata	Aves	Pelecaniformes	Ardeidae	Egretta	Egretta sp.	16
Metazoa	Chordata	Aves	Pelecaniformes	Phalacrocoracidae	Microcarbo	Microcarbo melanoleucos	40
Metazoa	Chordata	Aves	Pelecaniformes	Phalacrocoracidae	Phalacrocorax	Phalacrocorax carbo	18
Metazoa	Chordata	Aves	Pelecaniformes	Phalacrocoracidae	Phalacrocorax	Phalacrocorax sulcirostris	13
Metazoa	Chordata	Aves	Podicipediformes	Podicipedidae	Tachybaptus	Tachybaptus novaehollandiae	13
Metazoa	Chordata	Aves	Psittaciformes	Cacatuidae	Cacatua	Cacatua galerita	115
Metazoa	Chordata	Aves	Psittaciformes	Cacatuidae	Cacatua	Cacatua sp.	39
Metazoa	Chordata	Aves	Psittaciformes	Cacatuidae	Eolophus	Eolophus roseicapilla	30
Metazoa	Chordata	Aves	Psittaciformes	Psittacidae	Alisterus	Alisterus scapularis	11
Metazoa	Chordata	Aves	Psittaciformes	Psittacidae	Platycercus	Platycercus eximius	21
Metazoa	Chordata	Aves	Psittaciformes	Psittacidae	Psephotus	Psephotus haematonotus	18
Metazoa	Chordata	Aves	Psittaciformes	Psittacidae	Psittacula	Psittacula sp.	1
Metazoa	Chordata	Aves	Psittaciformes	Psittaculidae	Melopsittacus	Melopsittacus undulatus	1
Metazoa	Chordata	Aves	Psittaciformes	Psittaculidae	Trichoglossus	Trichoglossus sp.	13
Metazoa	Chordata	Aves	Strigiformes	Strigidae	Ninox	Ninox novaeseelandiae	2

Kingdom	Phylum	Class	Order	Family	Genus	Species	N site detections
Metazoa	Chordata	Aves	Strigiformes	Tytonidae	Tyto	Tyto alba	1
Metazoa	Chordata	Aves	Struthioniformes	Struthionidae	Struthio	Struthio sp.	1
Metazoa	Chordata	Chondrichthyes	Pristiophoriformes	Pristiophoridae	Pristiophorus	Pristiophorus sp.	1
Metazoa	Chordata	Hyperoartia	Petromyzontiformes	Geotriidae	Geotria	Geotria australis	18
Metazoa	Chordata	Lepidosauria	Squamata	Scincidae	Carinascincus	Carinascincus sp.	2
Metazoa	Chordata	Lepidosauria	Squamata	Scincidae	Eulamprus	Eulamprus heatwolei	4
Metazoa	Chordata	Lepidosauria	Squamata	Scincidae	Eulamprus	Eulamprus tympanum	2
Metazoa	Chordata	Lepidosauria	Squamata	Scincidae	Lampropholis	Lampropholis delicata	1
Metazoa	Chordata	Lepidosauria	Squamata	Scincidae	Lampropholis	Lampropholis guichenoti	12
Metazoa	Chordata	Lepidosauria	Squamata	Scincidae	Morethia	Morethia sp.	2
Metazoa	Chordata	Lepidosauria	Squamata	Scincidae	Saproscincus	Saproscincus mustelinus	2
Metazoa	Chordata	Lepidosauria	Squamata	Scincidae	Tiliqua	Tiliqua scincoides	2
Metazoa	Chordata	Mammalia	Artiodactyla	Bovidae	Bos	Bos taurus	369
Metazoa	Chordata	Mammalia	Artiodactyla	Bovidae	Capra	Capra hircus	13
Metazoa	Chordata	Mammalia	Artiodactyla	Bovidae	Ovis	Ovis aries	178
Metazoa	Chordata	Mammalia	Artiodactyla	Cervidae	Cervus	Cervus elaphus	12
Metazoa	Chordata	Mammalia	Artiodactyla	Cervidae	Dama	Dama dama	20
Metazoa	Chordata	Mammalia	Artiodactyla	Cervidae	Rusa	Rusa unicolor	271
Metazoa	Chordata	Mammalia	Artiodactyla	Suidae	Sus	Sus scrofa	113
Metazoa	Chordata	Mammalia	Carnivora	Canidae	Canis	Canis lupus	213
Metazoa	Chordata	Mammalia	Carnivora	Canidae	Vulpes	Vulpes vulpes	91
Metazoa	Chordata	Mammalia	Carnivora	Felidae	Felis	Felis catus	60
Metazoa	Chordata	Mammalia	Chiroptera	Pteropodidae	Pteropus	Pteropus poliocephalus	1
Metazoa	Chordata	Mammalia	Chiroptera	Vespertilionidae	Chalinolobus	Chalinolobus sp.	2
Metazoa	Chordata	Mammalia	Dasyuromorphia	Dasyuridae	Antechinus	Antechinus sp.	7
Metazoa	Chordata	Mammalia	Diprotodontia	Acrobatidae	Acrobates	Acrobates pygmaeus	6

Kingdom	Phylum	Class	Order	Family	Genus	Species	N site detections
Metazoa	Chordata	Mammalia	Diprotodontia	Macropodidae	Macropus	Macropus fuliginosus	3
Metazoa	Chordata	Mammalia	Diprotodontia	Macropodidae	Macropus	Macropus giganteus	100
Metazoa	Chordata	Mammalia	Diprotodontia	Macropodidae	Notamacropus	Notamacropus rufogriseus	8
Metazoa	Chordata	Mammalia	Diprotodontia	Macropodidae	Osphranter	Osphranter sp.	4
Metazoa	Chordata	Mammalia	Diprotodontia	Macropodidae	Wallabia	Wallabia bicolor	127
Metazoa	Chordata	Mammalia	Diprotodontia	Petauridae	Petaurus	Petaurus australis	2
Metazoa	Chordata	Mammalia	Diprotodontia	Petauridae	Petaurus	Petaurus breviceps	93
Metazoa	Chordata	Mammalia	Diprotodontia	Phalangeridae	Trichosurus	Trichosurus cunningham	37
Metazoa	Chordata	Mammalia	Diprotodontia	Phalangeridae	Trichosurus	Trichosurus vulpecula	359
Metazoa	Chordata	Mammalia	Diprotodontia	Phascolarctidae	Phascolarctos	Phascolarctos cinereus	37
Metazoa	Chordata	Mammalia	Diprotodontia	Potoroidae	Potorous	Potorous tridactylus	1
Metazoa	Chordata	Mammalia	Diprotodontia	Pseudocheiridae	Petauroides	Petauroides volans	9
Metazoa	Chordata	Mammalia	Diprotodontia	Pseudocheiridae	Pseudocheirus	Pseudocheirus peregrinus	126
Metazoa	Chordata	Mammalia	Diprotodontia	Vombatidae	Vombatus	Vombatus ursinus	71
Metazoa	Chordata	Mammalia	Lagomorpha	Leporidae	Lepus	Lepus europaeus	8
Metazoa	Chordata	Mammalia	Lagomorpha	Leporidae	Oryctolagus	Oryctolagus cuniculus	52
Metazoa	Chordata	Mammalia	Monotremata	Ornithorhynchidae	Ornithorhynchus	Ornithorhynchus anatinus	220
Metazoa	Chordata	Mammalia	Monotremata	Tachyglossidae	Tachyglossus	Tachyglossus aculeatus	5
Metazoa	Chordata	Mammalia	Peramelemorphia	Peramelidae	Isoodon	Isoodon obesulus	2
Metazoa	Chordata	Mammalia	Peramelemorphia	Peramelidae	Perameles	Perameles sp.	3
Metazoa	Chordata	Mammalia	Perissodactyla	Equidae	Equus	Equus caballus	8
Metazoa	Chordata	Mammalia	Primates	Hominidae	Homo	Homo sapiens	319
Metazoa	Chordata	Mammalia	Rodentia	Muridae	Hydromys	Hydromys chrysogaster	53
Metazoa	Chordata	Mammalia	Rodentia	Muridae	Mastacomys	Mastacomys fuscus	7
Metazoa	Chordata	Mammalia	Rodentia	Muridae	Mus	Mus musculus	40
Metazoa	Chordata	Mammalia	Rodentia	Muridae	Rattus	Rattus norvegicus	17

Kingdom	Phylum	Class	Order	Family	Genus	Species	N site detections
Metazoa	Chordata	Mammalia	Rodentia	Muridae	Rattus	Rattus sp.	221
Metazoa	Chordata	Reptilia	Testudines	Chelidae	Chelodina	Chelodina longicollis	33
Metazoa	Chordata	Reptilia	Testudines	Chelidae	Chelodina	Chelodina sp.	15
Metazoa	Chordata	Reptilia	Testudines	Chelidae	Chelodina	Chelodina expansa	27
Metazoa	Chordata	Reptilia	Testudines	Chelidae	Emydura	Emydura macquarii	8

Appendix 2. Project and eDNA method improvement

The scale of this project highlighted several areas of improvement that could be undertaken to ensure the integrity of data, and to limit false positive and false negative detections in samples or at sites:

1. Metadata collection and confidence in site sample location.

There were issues identified with the phone App used to collect metadata. Principally, if there was no network signal, the site coordinates did not auto populate correctly. There were also no contact details collected on individual citizen scientists, which made it difficult to correct these issues (which were also magnified by Covid-19 lockdowns and issues with Australia Post at the time of sampling). We recommend improvement of the App so that it works when offline from the network, collection of contact details of individual citizen scientists partaking in sampling, and some redundancy of recording sampling site location to ensure accuracy or potential follow-up with citizen scientists.

2. The amount of water filtered through individual filters.

The syringe-disc filters used for this project were 1.2 μM in pore size. General eDNA research suggests that 5 μM filters, which will enable greater volumes of water to be filtered due to the larger pore size, enable the capture of more DNA from vertebrate species and we suggest these are used for future citizen science-based projects. Similarly, a video made available in the App that shows the method and describes the minimum amount of water that needs to be filtered for effective capture of eDNA.

3. Elimination of DNA negative samples prior to metabarcoding using qPCR.

Due to the large number of samples processed for this project, we encountered issues when a high proportion of samples were negative in the metabarcoding process using the NextSeq Illumina platform. This is likely due to index switching or hopping (see below). This issue can be eliminated by screening samples first using qPCR for DNA concentration using general fish primers (Furlan & Gleeson 2016) and removing samples that have very low fish DNA concentrations.

4. Unique dual indexes (UDIs) for metabarcoding.

Index switching (index sequences initially assigned to a specific sample are incorrectly assigned to other samples in a pool of samples) is a common occurrence in Illumina sequencing technology, and generally occurs when free adapters are present within a sample library. This issue can also increase with read depth (e.g. when using platforms such as the NextSeq Illumina sequencing platform and achieving read depths >60,000 reads/sample). Similarly, contamination occurring during library construction can result in false positive samples. The use of unique dual indexes (UDIs) in the first round of PCR can eliminate issues of contamination during library construction (between first and second PCR, and sequencing) and reduce index switching to negligible levels. While there is a large initial upfront cost for UDIs to process samples in a project of this size (~4000 samples), we recommend they are used to avoid false positive sample issues.

5. Multiple amplicons for target groups

Recent research has shown that different amplicon assays can result in some biases in taxa that are detected for different groups (). In this project we used a Vertebrate assay that targeted the DNA of all vertebrates (aquatic and terrestrial), as that was the initial brief provided by Odonata (to capture data on both aquatic and terrestrial vertebrates). This may have led to some lower fish detections than expected (e.g. false negatives) because other terrestrial DNA was amplified in higher concentrations. We used a fish amplicon on some samples (using the UDI approach mentioned above), to try and improve detections in samples that had low (or no) fish detections. Given recent research, we recommend applying a multiple amplicon (assay) approach for intended target groups, which will likely lead to improved detections, and lower false negatives.

6. Reference sequences

The large spatial scale of this project highlighted some issues with reference sequence databases. While we had reference sequences for the majority of target aquatic species in our database, we did not have good representation across a species range (intraspecific diversity and how this relates to interspecific diversity for closely related species). Ideally, more reference sequences from some closely related species (e.g., blackfish, pygmy perch, galaxiids, gudgeons etc) would increase resolution and provide further clarity around the resolution within the Vertebrate amplicon.

Appendix 3. Interesting aquatic native species site detections.

Species	Basin	Latitude	Longitude	Comments
Maccullochella peelii	Wimmera-Avon Rivers	-36.4789	142.0478	Potential new detection or false positive
Pseudaphritis urvillii	Ovens River	-36.5829	146.8885	Potential new detection or false positive
Pseudaphritis urvillii	Goulburn River	-36.6863	145.5415	Potential new detection or false positive
Galaxias olidus sp. complex	South Gippsland	-38.4607	146.2337	Potential new detection
Galaxias olidus sp. complex	South Gippsland	-38.5179	146.2355	Potential new detection
Galaxias olidus sp. complex	South Gippsland	-38.6232	146.3062	Potential new detection
Galaxias olidus sp. complex	Latrobe River	-38.1004	146.2696	Potential new detection
Galaxias olidus sp. complex	Thomsen River	-37.8168	146.9469	Potential new detection
Galaxias olidus sp. complex	Thomsen River	-37.3969	146.7505	Potential new detection
Galaxias olidus sp. complex	Mitchell River	-37.1309	147.1801	Potential new detection
Galaxias olidus sp. complex	Mitchell River	-37.2277	147.2219	Potential new detection
Galaxias olidus sp. complex	Mitchell River	-37.3345	147.4973	Potential new detection
Galaxias olidus sp. complex	Tambo River	-37.2734	147.8567	Potential new detection
Galaxias olidus sp. complex	Tambo River	-37.2686	147.8863	Potential new detection
Galaxias olidus sp. complex	Tambo River	-37.2904	147.9545	Potential new detection
Galaxias olidus sp. complex	Snowy River	-37.752	148.339	Potential new detection
Galaxias olidus sp. complex	East Gippsland	-37.4043	148.8921	Potential new detection
Galaxias olidus sp. complex	East Gippsland	-37.4266	149.0076	Potential new detection
Galaxias olidus sp. complex	East Gippsland	-37.5062	148.8532	Potential new detection
Galaxias olidus sp. complex	East Gippsland	-37.51	148.8583	Potential new detection
Galaxias olidus sp. complex	East Gippsland	-37.5488	148.798	Potential new detection
Galaxias olidus sp. complex	East Gippsland	-37.5664	149.1468	Potential new detection
Galaxias olidus sp. complex	East Gippsland	-37.7071	149.1691	Potential new detection
Galaxiella pusilla	South Gippsland	-38.9753	146.2777	Has been recorded here for >20 years
Gobiomorphus coxii	Snowy River	-37.4427	148.1578	Upstream of last known detection
Gobiomorphus coxii	Snowy River	-37.5024	148.2028	Downstream of last known detection
Gobiomorphus coxii	Snowy River	-37.5185	148.2638	Downstream of last known detection
Hypseleotris spp.	East Gippsland	-37.4266	149.0076	Possible false positive or new detection
Hypseleotris spp.	Tambo River	-37.1116	147.8128	Possible false positive or new detection
Hypseleotris spp.	Tambo River	-37.3803	148.0971	Possible false positive or new detection
Hypseleotris spp.	Mitchell River	-37.7963	147.546	Possible false positive or new detection
Hypseleotris spp.	Barwon River	-38.0561	144.1616	Possible false positive or new detection
Nematalosa erebi	Yarra River	-37.6738	144.9383	Likely false positive
Neochanna cleaveri	South Gippsland	-38.9274	146.2783	Potential new basin detection
Neochanna cleaveri	South Gippsland	-38.9751	146.2774	Potential new basin detection
Neochanna cleaveri	South Gippsland	-39.026	146.3325	Potential new basin detection
Neochanna cleaveri	South Gippsland	-38.9768	146.4371	Potential new basin detection
Neochanna cleaveri	South Gippsland	-39.0211	146.4349	Potential new basin detection
Philypnodon macrostomus	South Gippsland	-38.9768	146.4371	Potential new basin detection
Philypnodon macrostomus	South Gippsland	-38.2674	146.9224	Potential new basin detection
Philypnodon macrostomus	South Gippsland	-38.2926	147.1553	Potential new basin detection
Philypnodon macrostomus	Goulburn River	-37.0846	145.6125	Likely false positive
Tandanus tandanus	Yarra River	-37.6226	145.1924	Not known from this basin
Tandanus tandanus	Glenelg River	-37.4414	141.2735	Possible new detection in new basin
Tandanus tandanus	Millicent Coast	-36.3431	140.9812	Possible new detection in new basin
Galaxias brevipinnis	Loddon River	-36.3241	143.8646	Possible false positive
Galaxias brevipinnis	Campaspe River	-36.5607	144.5866	Possible false positive
Galaxias brevipinnis	Campaspe River	-37.3869	144.4509	Possible false positive
Galaxias maculatus	Broken River	-36.0991	145.4797	Possible false positive

Galaxias maculatus	Goulburn River	-36.7602	145.5611	Possible false positive
Galaxias maculatus	Goulburn River	-37.016	145.1357	Possible false positive
Galaxias maculatus	Goulburn River	-37.2652	144.9446	Possible false positive
Galaxias maculatus	Ovens River	-36.061	146.3086	Possible false positive
Galaxias maculatus	Ovens River	-36.22	146.7293	Possible false positive
Galaxias maculatus	Ovens River	-36.5829	146.8885	Possible false positive
Galaxias maculatus	Upper Murray River	-36.2245	147.1499	Possible false positive