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Development and evaluation of fish eDNA metabarcoding assays facilitate the detection of cryptic seahorse taxa (family: Syngnathidae)

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Abstract

Environmental DNA (eDNA) metabarcoding methods have demonstrated their potential as noninvasive techniques for the monitoring and conservation of marine fishes, including rare and endangered taxa. However, the majority of these investigations have focused on large-bodied taxa such as sharks and sturgeons. In contrast, eDNA studies on small-bodied cryptic taxa are much less common. As a case in point, seahorses (members of the Syngnathidae family) have never been detected by eDNA, despite the fact that globally there are 14 species classified as "Threatened" by the IUCN. Here, we critically evaluate the ability of two existing broad-spectrum fish metabarcoding assays (MiFish and 16S Fish) and explore the efficacy of two newly designed fish metabarcoding assays (16S_FishSyn_Short and 16S_FishSyn_Long) to detect Syngnathidae amidst a wide spectrum of fish species. Furthermore, a custom Western Australian 16S rRNA fish database was created to increase the likelihood of correct taxonomic assignments. With the newly designed assays, we detected four Syngnathidae species in a targeted eDNA survey of the Perth metropolitan area (Western Australia). These detections include the seahorse species *Hippocampus subelongatus* and *Hippocampus breviceps*, which represents the first time seahorse species have been detected using eDNA. The existing MiFish and 16S Fish assays did not detect any Syngnathidae. This evaluation of all four fish metabarcoding assays reinforces the view that every PCR assay has "blind spots". In the context of complex environmental samples, no assay is universal and false negatives will occur due to a combination of PCR efficacy, primer binding, assay sensitivity, degeneracies in the primers, template competition, and amplicon length. Taken together, these data indicate that eDNA methodologies, with ongoing optimizations, will become an integral part of monitoring small-bodied cryptic taxa such as seahorses, gobies, and blennies and can assist in mapping species' distributions and prioritizing conservation areas.

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KEYWORDS
biodiversity, biomonitoring, conservation, cryptobenthic, environmental DNA, metabarcoding, mitochondrial DNA, seahorse

1 | INTRODUCTION

Biodiversity loss is a major societal and economic concern (Cardinale et al., 2012), with increasing anthropogenic pressures and climate change resulting in a continuous decline of global biodiversity and ecosystem health (Butchart et al., 2010). To detect changes in ecological communities, monitoring programs need to be sensitive and provide accurate data on species presence/absence and their distribution (Baker et al., 2016). These data are often difficult to obtain in aquatic environments using traditional methods due to the reduced accessibility or visibility of the environment, the occurrence of rare and cryptic species (Jerde, Mahon, Chadderton, & Lodge, 2011), limited taxonomic expertise (Hopkins & Freckleton, 2002), and funding limitations (Lundquist & Granek, 2005). Rare aquatic species are, by nature, difficult to monitor and study in their marine or estuarine environments (Pikitch, 2018). For these taxa, knowledge regarding species richness at a particular location, range distribution, and population size is often incomplete or "data deficient" (Niemiller et al., 2017). Rapid advances in DNA sequencing technologies offer the opportunity to generate high-quality biodiversity data with increased sensitivity (Bourlat et al., 2013; Thomsen et al., 2012), rapidly advancing the current scope of aquatic ecosystem monitoring (Bush et al., 2019).

One approach that is transforming the way aquatic ecosystems are monitored is through environmental DNA (eDNA, i.e., genetic material derived from whole microscopic organisms or shed from multicellular organisms (DiBattista et al., 2017; Taberlet, Coissac, Hajibabaei, & Rieseberg, 2012)), which can be obtained from environmental samples, such as water, sediment, and soil, and used to pinpoint species distributions. An extension of this approach is eDNA metabarcoding, which specifically refers to the simultaneous detection of multiple species through the design and application of taxonomically broad PCR-based assays. When applied to environmental samples, it can characterize species assemblages of whole ecological communities (Deiner et al., 2017; Hansen, Bekkevold, Clausen, & Nielsen, 2018; Taberlet, Bonin, Zinger, & Coissac, 2018). The utility of eDNA is vast, with research demonstrating increased taxonomic resolution (Lim et al., 2016; Sigsgaard, Carl, Møller, & Thomsen, 2015; Valentini et al., 2016) and greater sensitivity in the detection of rare and invasive species (Dejean et al., 2012; Jerde et al., 2011; Piaggio et al., 2014) compared to traditional monitoring methods. Furthermore, by avoiding the need for visual observation, capture, and direct sampling (Goldberg et al., 2016), eDNA surveys frequently overcome some of the cost, time, biases, and at times invasive nature associated with traditional monitoring methods (Jeunen et al., 2019; Thomsen & Willerslev, 2015).

eDNA metabarcoding surveys have been applied to a wide range of aquatic ecosystems, including: rivers (Bylemans, Gleeson, Hardy, & Furlan, 2018; Bylemans, Gleeson, Lintermans, et al., 2018); lakes (Fujii et al., 2019); open ocean (Truelove, Andruszkiewicz, & Block, 2019); coastal habitats (Andruszkiewicz et al., 2017; Koziol et al., 2019); and reefs (DiBattista et al., 2017; West et al., 2020)). These surveys primarily use broad-spectrum fish metabarcoding assays 16S Fish (Berry et al., 2017; Deagle et al., 2007) and MiFish-U (Miya et al., 2015), which respectively target the 16S and 12S rDNA regions of the mitochondrial genome. Despite the frequent use of 16S Fish and MiFish-U as broad-spectrum fish metabarcoding assays, there has been no critical comparison of the taxon range and sensitivities of these assays on a set of diverse environmental samples. These comparative studies are needed when planning an eDNA experimental design to understand which metabarcoding assays are most suitable. Furthermore, no studies using MiFish-U or 16S Fish have reported the detection of Syngnathidae taxa (pipefishes, seahorses, and seadragons) as of present.

The Syngnathidae family is comprised of almost 300 marine, brackish, and freshwater species distributed globally (Wilson & Orr, 2011). Syngnathidae are extremely vulnerable to human impact and subsequent population declines due to life history traits such as low fecundity, restricted distributions, high rates of endemism, and limited mobility (Foster & Vincent, 2004; Shokri, Gladstone, & Jelbart, 2009). Many Syngnathidae species are considered threatened (7.2%; IUCN, 2019) with population declines attributed to exploitation for the aquarium trade, Traditional Chinese Medicines, habitat degradation, and as bycatch in commercial trawl fisheries (Lourie, 2000; Luo, Qu, Li, Wang, & Lin, 2015; Martin-Smith & Vincent, 2006). However, many more species (over 30%) lack the data necessary to assess their extinction risk (IUCN, 2019). With the risk of a "silent extinction" for many Syngnathidae species, the design of a noninvasive method for monitoring and managing these cryptic species may be critical to their survival.

False negatives (failure to detect a species when they are in fact present) are significant in the management of threatened species (Delaney & Leung, 2010; Ficetola et al., 2014; Furlan & Gleeson, 2017). For this reason, we aimed to determine whether the Syngnathidae family are being inadvertently omitted by current broad-spectrum fish assays. Australia is home to 128 species of Syngnathidae in 40 genera, 65 of which are found in Western Australian waters (Bray, 2019). The Perth metropolitan area in Western Australia was chosen as our study site as it encompasses several habitat types, including brackish and salt water (Kendrick & Hyndes, 2003). To increase the likelihood of correct taxonomic assignment across a broad range of bony fish taxa, a custom 16S rRNA fish database was created using specimens collected along the coastline of Western Australia. The primary objective of this study was therefore to evaluate and optimize a set of broad-spectrum fish eDNA metabarcoding assays capable of detecting Syngnathidae in

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TABLE 1 Existing fish eDNA metabarcoding assays and newly designed assays used throughout this study

Assays	Gene region	Amplicon length (bp)	Primer sequence (5'>3')	GC content (%)	Annealing temperature (°C)	Reference
16S Fish forward	16S	~200	GACCCTATGGAGCTTTAGAC	45	54	Berry et al., 2017[40]
16S Fish reverse			CGCTGTTATCCCTADRGTAACT	50		Deagle et al., 2007[39]
MiFish-U forward	12S	~170	GTCGGTAAAACTCGTGCCAGC	57.1	56	Miya et al., 2015[16]
MiFish-U reverse			CATAGTGGGGTATCTAATCCCAGTTTG	44.4		
16S_FishSyn_Short forward	16S	~80	GACGAGAAGACCCTGTGGAGC	61.9	55	This study
16S_FishSyn_Short reverse			CCGYGGTCGCCCCAAC	80		
16S_FishSyn_Long forward	16S	~200	GACGAGAAGACCCTDTGGAG	57.9	55	This study
16S_FishSyn_Long reverse			GRATTGCGNTGTTATCCCT	47.1		

environmental samples in the context of other fish assemblages. In addition, we critically evaluated four fish metabarcoding assays across a set of diverse environmental samples, to better understand the strengths, weaknesses, and limitations of each assay in isolation or when used in combination.

2 | METHODS

2.1 | Western Australian 16S rRNA fish database curation

Curated databases for select gene regions enhance the utility and taxonomic range of current metabarcoding databases (Deiner et al., 2017). A custom 16S rRNA fish database was created using a combination of targeted sampling and subsamples provided and taxonomically identified by the West Australian Department of Primary Industries and Regional Development (DPIRD) for target and bycatch species (Table S1). Tissue samples from 303 vouchered fish species were removed from storage buffer (20% salt-saturated DMSO or 95% ethanol) and dried before subsampling with a target weight of 20 mg. Extraction of DNA from the subsampled tissues was automated using the QIAcube extraction platform (Qiagen) and the DNeasy Blood and Tissue Kit (Qiagen) with modified protocols. DNA extracts were quantified using the NanoDrop™ 2000 spectrophotometer (Thermo Fisher). Samples were pooled (utilizing DNA quantification results) for sequencing by taxonomic family, with no species from identical families within the same sequencing pool. This reduced the chances of taxonomic ambiguity between closely related species during bioinformatic analysis due to sequencing error. Metabarcoding and next-generation sequencing (NGS) were performed as below and a reference haplotype for each species was determined. These sequences were deposited into GenBank under the accession MN473514 to MN473874.

2.2 | Primer design and validation

Several PCR metabarcoding assays were designed to target the 16S rRNA region of the mitochondrial genome of Syngnathidae species and species of fish typically detected using the 16S Fish assay. Publicly available (NCBI) Syngnathidae and other fish 16S reference sequences were aligned using Geneious v. 10.2.6 to identify short conserved regions capable of amplifying degraded DNA commonly encountered in environmental samples (Tables S2 and S3. Assays were designed based on guidelines specified in a previous study (Bustin & Huggett, 2017). In brief, the assays were free from secondary structures, had balanced GC content and similar annealing temperatures on forward and reverse primers.

To determine efficacy, the newly developed assays and MiFish-U and 16S Fish were tested in silico (Figure S1) and in vitro through

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quantitative PCR (qPCR) of tissue and environmental samples. Reactions were performed using neat and a three-point ten-fold dilution series of single-source *H. subelongatus* tissue (obtained from De Brauwer et al., 2019). The assays were further tested on aquarium water that held *H. subelongatus*, among other species, from the Aquarium of Western Australia to determine their ability to detect Syngnathidae taxa in controlled environmental samples. Tissue and filtered aquarium water were extracted and amplified as described below. Two optimal assay sets (termed 16S_FishSyn_Short and 16S_FishSyn_Long) were selected for further testing based on their reliability to amplify Syngnathidae mtDNA in tissue and aquarium samples. These assays were used, in conjunction with the 16S Fish and MiFish-U assays, throughout the remainder of the study (Table 1).

2.3 | Site description and sample collection

Sampling was conducted over a week period in May 2018, in Perth, Western Australia (Table S4). The southwest region of Western Australia is known for its unique fish assemblages and high rates of endemism (Melville-Smit, Larsen, de Graaf, & Lawrence, 2010; Richards et al., 2016). The temperate, coastal waters of the Perth metropolitan region were chosen for this study as several Syngnathidae species inhabit this area, including *H. subelongatus*, *Stigmatopora argus*, and *Filicampus tigris* (Kendrick & Hyndes, 2003). Samples were collected from five sites: (a) Bicton Baths, (b) Blackwall Reach, (c) Ammo Jetty, (d) Rockingham Wreck Trail, and (e) Mt Henry Bridge (Figure 1). These specific sites were chosen as Syngnathidae presence was confirmed through recent recreational diver observations.

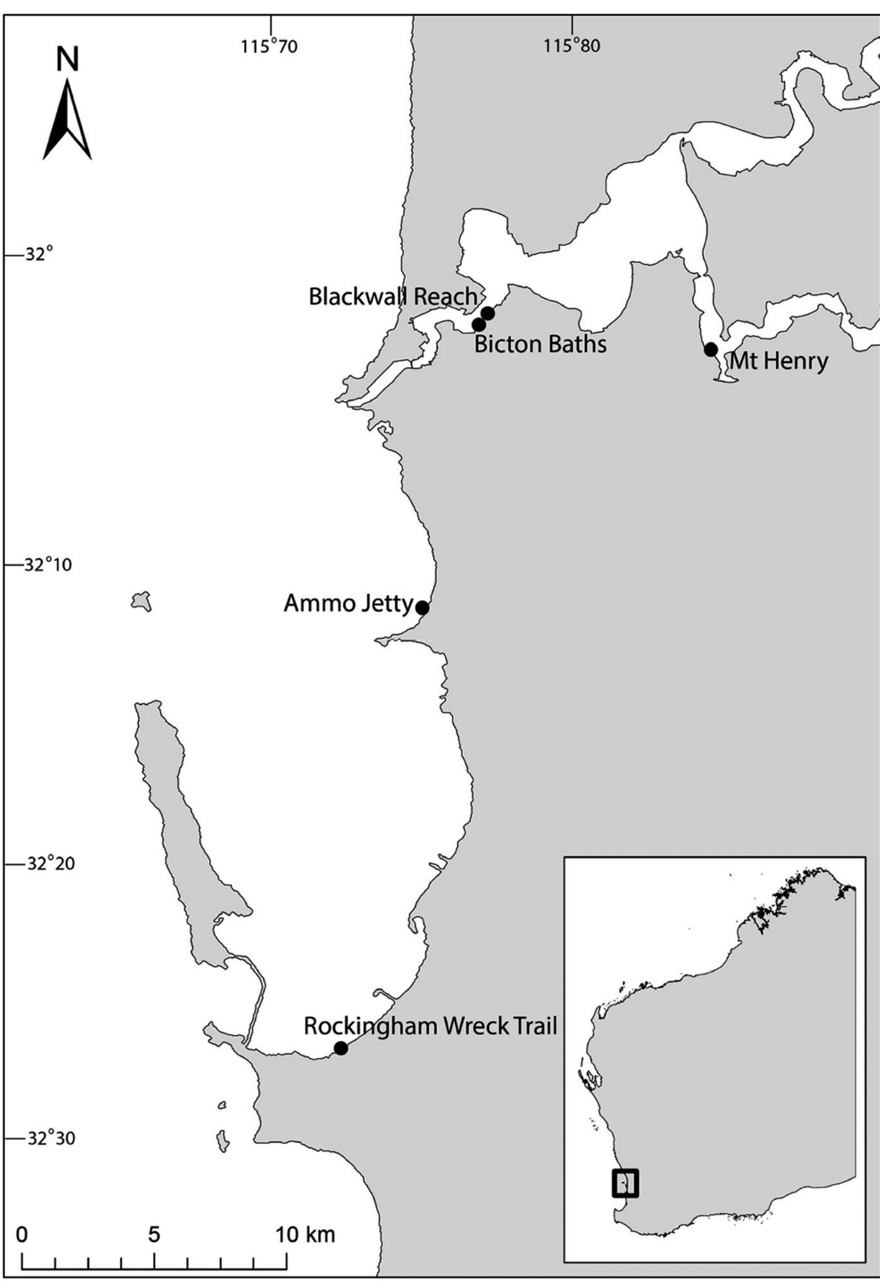

FIGURE 1 Map of field sites (Mt Henry, Bicton Baths, Blackwall Reach, Ammo Jetty, and Rockingham Wreck Trail) in the Perth metropolitan area of Western Australia. Created with ArcGIS (ESRI, 2011)

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Five replicate surface (0–2 m deep) and bottom water (7–12 m deep) samples were collected via scuba and snorkeling at each site (*n* = 50) as a means of empirically evaluating the effects of depth selection on the detection rates of each assay. Water samples (1 L per replicate) were collected using sterile Nalgene bottles that were opened underwater at the sampling site and then immediately closed following sampling. Water samples were filtered using a Sentino peristaltic pump onto 47-mm filter membranes (Pall Life Sciences) within four hours of sampling to minimize DNA degradation. A 0.22 µm pore size was used for ocean sites (Ammo Jetty and Rockingham Wreck Trail) and 0.45 µm pore size for river sites due to increased turbidity in these environments (Bicton Baths, Blackwall Reach and Mt Henry). Filter membranes were frozen at −20°C until DNA extraction.

2.4 | DNA extraction and metabarcoding

Water samples were extracted using a DNeasy Blood and Tissue kit (Qiagen) and a modified protocol (Stat et al., 2018). Extraction controls (i.e., no sample) were implemented for every site and extracted alongside the water samples. Metabarcoding was performed in duplicate on each DNA extract and control for each assay (Table 1). qPCR reactions (25 ml) consisted of the following: 2.5 mM/L $MgCl_2$ (Applied Biosystems), 1× PCR Gold buffer (Applied Biosystems), 1 U AmpliTaq Gold DNA polymerase (Applied Biosystems), 0.25 mM/L dNTPs (Astral Scientific, Australia), 0.4 mg/ml bovine serum albumin (Fisher Biotec, Australia), 0.4 µM forward and reverse primer (specified in Table 1), 0.6 µl of a 1:10,000 solution of SYBR Green dye (Life Technologies), and 4 µl of template DNA. qPCR amplifications were performed on a StepOnePlus Real-Time PCR System (Applied Biosystems). To reduce the likelihood of index-tag switching and chimera production, multiple unique forward and reverse fusion tag combinations were added to the qPCR products, each consisting of an adapter sequence, gene-specific primers, and a unique multiple identifier (MID). A "no template" control was also included in each qPCR to detect any cross-contamination between samples (*n* = 16). Additionally, a positive control (*Hippocampus subelongatus* tissue) was used in duplicate for each primer set (Table 1). Thermocycler conditions were as follows: 95°C for 5 m, 50 cycles of 95°C for 30 s, annealing temperature of 54°C (16S Fish), 55°C (16S_FishSyn_Short and 16S_FishSyn_Long), and 60°C (MiFish), completed by a 72°C elongation step. Extraction and negative controls showed no sign of amplification and were therefore excluded from downstream analyses.

Resulting amplicons were pooled in approximate equimolar ratios, size-selected (150-450bp) using a Pippin Prep (Sage Science), and purified using a QIAquick PCR Purification Kit (Qiagen). The final library was quantified using a QIAxcel Advanced System (Qiagen) and a Qubit Fluorometric Quantitation machine (Thermo Fisher) and sequenced on an Illumina Miseq platform using a 300 cycle Miseq V2 Reagent Kit and custom sequencing primers at Curtin University in Perth, Western Australia. Raw sequence data were deposited into GenBank under the accession SRX6841776.

2.5 | Bioinformatics and taxonomic assignment

Sequences with 100% matches to Illumina sequencing adapters, index barcodes, and template-specific primers were retained for downstream analysis using Geneious v. 10.2.6. USEARCH v. 10 (Edgar, 2010) was used to quality filter and discard reads with error rates of 1%, short reads (<50 bp), and chimeras. Resulting sequences were dereplicated into unique sequences and denoised into zero-radius operational taxonomic units (ZOTUs; denoised OTUs that aim to report correct biological sequences at a higher resolution than OTUs (Callahan, McMurdie, & Holmes, 2017; Edgar, 2016)). To remove erroneous ZOTUs caused by co-occurrence error, the LULU algorithm (Frøslev et al., 2017) was applied using R Studio v. 1.2.1335 (RStudio Team, 2015). ZOTUs were compared to a GenBank (NCBI) reference database and the custom Western Australian 16S fish database using the Basic Local Alignment Search Tool for nucleotides (BLASTn) on the Zeus system (Pawsey Supercomputing Centre).

ZOTUs with BLASTn parameters of E value above 10^{-5}, percentage identity below 94%, and query coverage per subject below 99% were removed to decrease uncertainty surrounding ZOTU taxonomic assignment (Alberdi, Aizpurua, Gilbert, & Bohmann, 2018; Porter & Hajibabaei, 2018; Xiong & Zhan, 2018). Taxonomic identities of ZOTUs were assigned and visualized in MEGAN v6 (METaGenome ANalyzer; Huson et al., 2016) using the LCA (lowest common ancestor) parameters: min bit score 100.0 and reports restricted to the top 10% of matches. To be conservative, taxonomic assignments were further evaluated against knowledge of species distributions ("Fishes of Australia," 2019). The output from MEGAN v6 was exported to Geneious v. 10.2.6 to create the phylogram.

2.6 | Statistical analysis

PRIMER v. 7 (Clarke & Gorley, 2015) was used to compare the effects of assay and site on taxonomic composition. Data were transformed into presence–absence format, and a Jaccard resemblance matrix (Schaalje & Beus, 1997) was created. A permutational multivariate analysis of variance (PERMANOVA) with factors site (Bicton Baths, Blackwall Reach, Ammo Jetty, Rockingham Wreck Trail, and Mt Henry) and assay (16S Fish, MiFish-U, 16S_FishSyn_Short and 16S_FishSyn_Long) was performed using the PERMANOVA + add on in PRIMER v. 7 (Anderson, 2001, 2005). All PERMANOVA tests were conducted using unrestricted permutation of raw data and 9,999 permutations. In the presence of significant effects, pairwise comparisons using PERMANOVA were performed to determine where the significant differences occurred. To visualize patterns in the data, nonmetric multidimensional scaling (nMDS) plots and canonical analysis of principal coordinates (CAP) plots were generated in PRIMER (Anderson, 2005) with the PERMANOVA + add on. Using the vegan package in R v. 3.6.0

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and RStudio v. 1.2.1335 (Oksanen et al., 2018; R Core Team, 2019; RStudio Team, 2015), rarefaction curves were generated to confirm adequate sequencing depth (Figure S2).

3 | RESULTS

3.1 | In silico and in vitro evaluation of fish metabarcoding assays

Existing fish metabarcoding assays (MiFish-U and 16S Fish) and newly developed fish metabarcoding assays (16S_FishSyn_Short and 16S_FishSyn_Long) were evaluated in vitro and in silico to infer their ability to detect Syngnathidae and more broadly fish taxa.

The MiFish-U assay failed to amplify control seahorse tissue from *H. subelongatus*, whereas the 16S Fish assay returned a weak (but positive) amplification. Both existing assays detected the bulk of fish species present in the control aquarium samples, however neither detected *H. subelongatus*. In silico analyses, using reference 12S and 16S Syngnathidae sequences, revealed that the existing assays likely performed poorly due to primer mismatches (Figure S1). 16S_FishSyn_Short and 16S_FishSyn_Long successfully amplified *H. subelongatus* from both the seahorse tissue and aquarium samples, with additional fish taxa detected in the latter (Table S5). To further evaluate the efficacy of these assays to detect fish, including Syngnathidae, a survey was conducted in the Perth metropolitan area.

3.2 | Evaluation of fish metabarcoding assays based on seawater collected in the Perth metropolitan region

A total of 4.6 million metabarcoding reads were obtained across the four fish assays after quality filtering. All assays reached asymptote in the rarefaction analyses. Assay selection significantly altered the taxonomic composition and number of species detected (*Pseudo-F*(1–3) = 33.865, $p < .001$), with pairwise comparisons indicating all assays were significantly different from one another ($p < .001$). The differences in community structure between the assays are visualized through the CAP plot (Figure 2).

A total of 68 fish species were detected with the four applied assays. 16S_FishSyn_Short was the best performing assay detecting 52 of 68 (76.5%) species, followed by 16S_FishSyn_Long with 43 of 68 (63.2%) species detections. In comparison, 16S Fish and MiFish detected 38 of 68 (55.9%) and 22 of 68 (32.4%) fish species, respectively. Relative to the best performing assay (16S_FishSyn_Short), the combination of all four assays resulted in 23.5% more species-level detections. It should be noted that lack of a regional fish 12S reference database was a contributing factor in the MiFish assignments (see data below).The taxonomic specificities and deficiencies of each assay are highlighted in Figure 3. Fish assemblages across each site displayed significant differences in the species that were detected (Figure 4; *Pseudo-F*(1–4) = 21.963, $p < .001$). This difference was further validated through pairwise comparisons

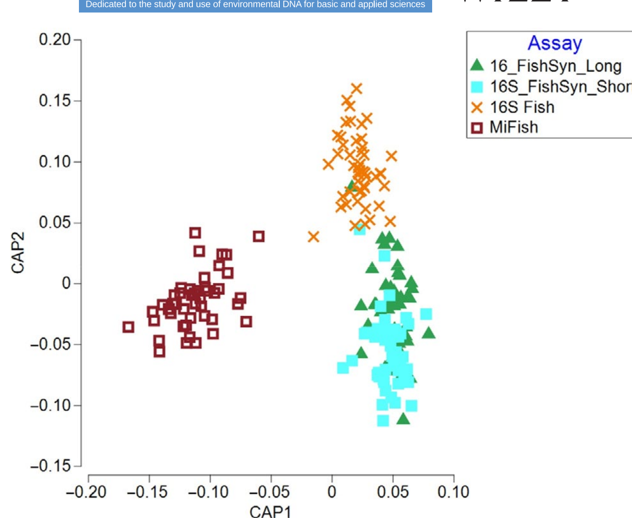

FIGURE 2 Canonical analysis of principal coordinates plot using a presence/absence transformed (Jaccard) data set of fish families detected (Perth, Western Australia). Samples are classified by assay used with distance between samples representing similarity in biological assemblage

indicating all sites were significantly different from one another ($p < .001$).

Only two (16S_FishSyn_Short and 16S_FishSyn_Long) of the four applied assays successfully detected Syngnathidae species at the Perth metropolitan sites (Figure 5). A total of four species of Syngnathidae were detected with these newly developed metabarcoding assays: *Hippocampus subelongatus* (Rockingham Wreck Trail), *Hippocampus breviceps* (Bicton Baths), *Stigmatopora argus* (Bicton Baths, Blackwall Reach and Ammo Jetty), and *Filicampus tigris* (Ammo Jetty). Both of the assays developed in this study successfully detected the two pipefish species, *Stigmatopora argus* and *Filicampus tigris*. However, each assay detected a unique seahorse species with 16S_FishSyn_Short detecting *Hippocampus subelongatus* and 16S_FishSyn_Long detecting *Hippocampus breviceps*. Furthermore, the frequency of detection was low, with only 0.02% of the total quality filtered reads assigned to Syngnathidae.

Through our empirical evaluation of the effects of depth selection on the detection rates of each assay, we detected more fish species per assay on average in bottom water (mean = 44.25 ± 0.95) than in surface water (mean = 43.25 ± 1.23; $p < .001$). Furthermore, bottom water detected more Syngnathidae species ($n = 4$) than surface water ($n = 2$), with seahorse species only detected in bottom water samples.

3.3 | Performance of the Western Australian 16S rRNA fish database using seawater collected in the Perth metropolitan region

The use of the Western Australian 16S rRNA fish database provided an additional two species assignments for the 16S gene region:

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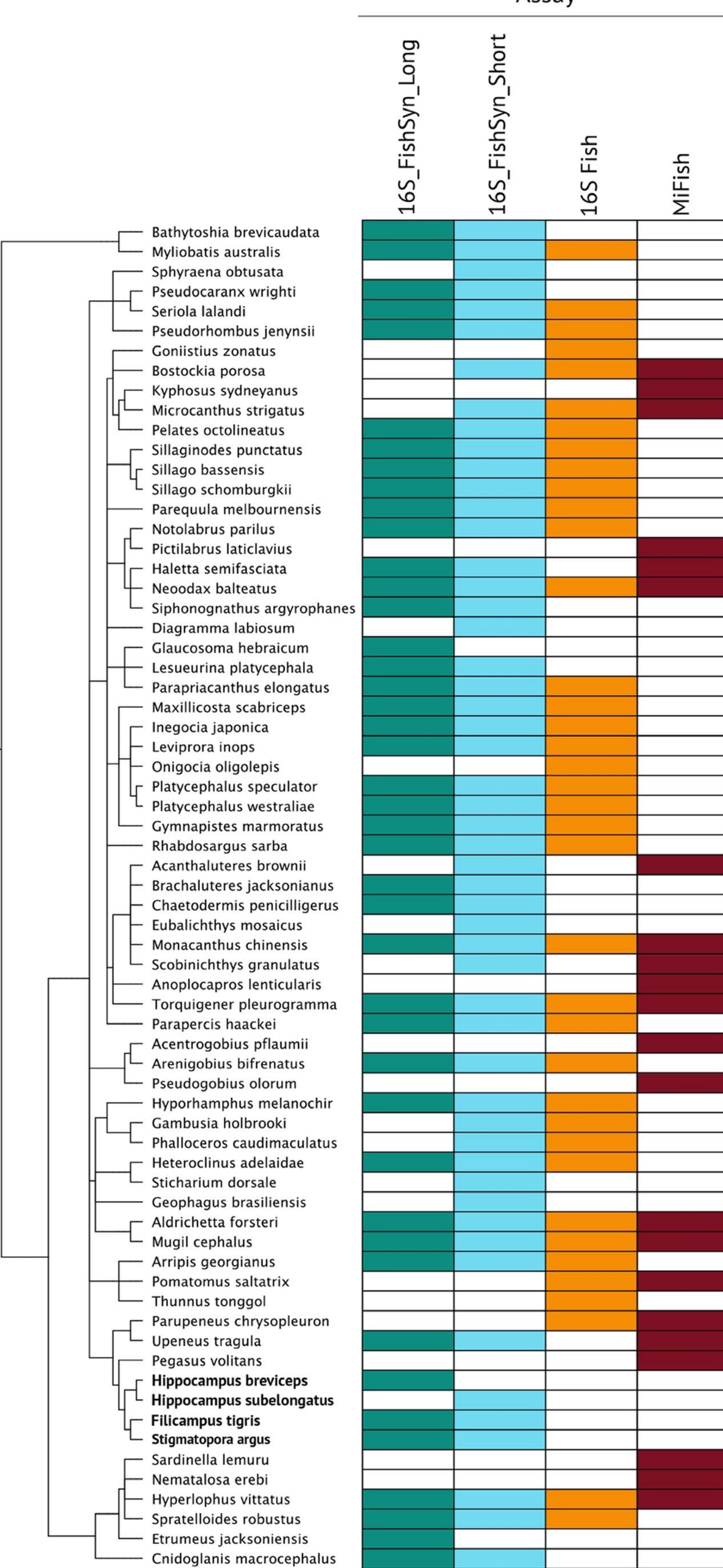

FIGURE 3 Taxonomic phylogram of total fish families detected across five locations in the Perth metropolitan region of Western Australia. Taxonomic detections of each assay are indicated through the coloured dots displayed in the legend

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Onigocia oligolepis and *Goniistius gibbosus*, and one additional genus assignment: *Leidotrigla*. Moreover, the use of the custom database resulted in better resolution by resulting in more (*N* = 3) species assignments for the 16S gene region. Of the 46 species that were not detected using the MiFish-U assay, 26 (56.5%) of these were due to mismatches and 20 (43.5%) were due to missing 12S rRNA sequences in the NCBI database. For the 16S Fish assay, 30 species were not detected with 28 (93.35%) of these due to mismatches and 2 (6.65%) due to missing 16S gene region sequences in the NCBI and custom fish database. The 16S_FishSyn_long assay failed to detect 25 species, of these 21 (84%) were due to mismatches and 4 (16%) were due to missing 16S gene region sequences. Of the 16 species not detected by the 16S_FishSyn_short assay, 12 (75%) of these were due to mismatches and 4 (25%) were due to missing 16S rRNA sequences.

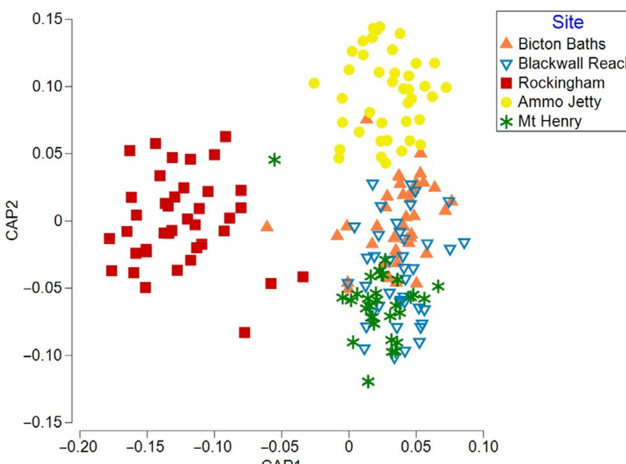

FIGURE 4 Canonical analysis of principal coordinates plot using a presence/absence transformed (Jaccard) data set of fish families detected (Perth, Western Australia). Samples are classified by site with distance between samples representing similarity in biological assemblage

4 | DISCUSSION

The metabarcoding assays MiFish-U (Miya et al., 2015) and 16S Fish (Berry et al., 2017; Deagle et al., 2007) are widely used throughout the eDNA literature as broad-spectrum fish metabarcoding assays as they target a taxonomically diverse range of fish species (DiBattista et al., 2017; Fujii et al., 2019; Miya et al., 2015; Stat et al., 2018). In this study, we demonstrated that these assays are inadvertently omitting Syngnathidae taxa in their detections due to primer binding factors. These assays may also be confounded by the low biomass of Syngnathidae relative to the other (more abundant) fish taxa. We subsequently developed two new fish metabarcoding assays capable of Syngnathidae detection and evaluated the taxon detections of these four assays in a varied set of environmental samples. While all aquatic environments differ in their composition, relative biomass, and genetic background, we hope the taxonomic strengths and weaknesses discovered in these metabarcoding experiments might aid in assay selection and experimental design in future eDNA studies.

When considered holistically, there were clear differences in assay performance, with 16S_FishSyn_Short and 16S_FishSyn_long detecting 52 of 68 and 43 of 68 fish species, respectively, while 16S Fish and MiFish detected 38 of 68 and 23 of 68 fish species, respectively. The newly developed metabarcoding assays detected 18 fish species that 16S Fish and MiFish did not. Among these undetected species were the West Australian dhufish (*Glaucosoma hebraicum*), the flathead sandfish (*Lesueurina platycephala*), the slender snake blenny (*Sticharium* dorsale), and the smooth stingray (*Bathytoshia*

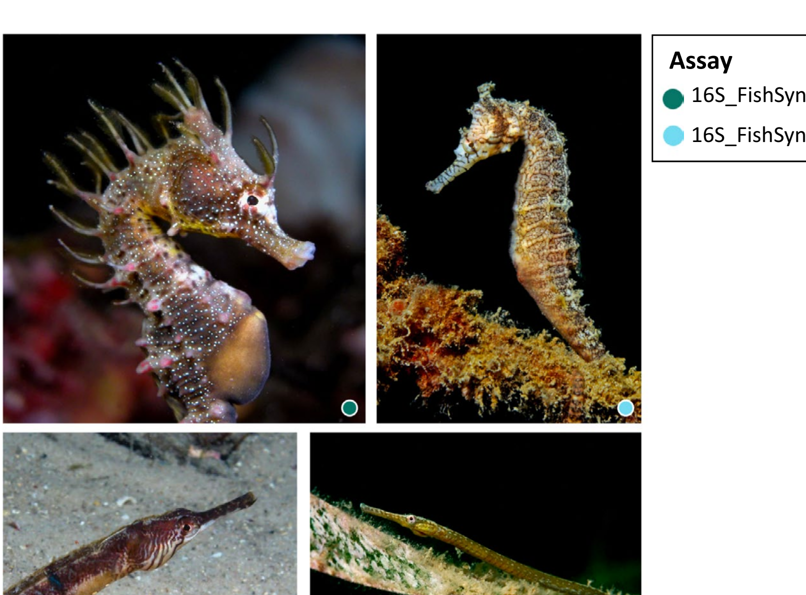

FIGURE 5 Total Syngnathidae taxa (*N* = 4) detected in this metabarcoding study across five locations in the Perth metropolitan region of Western Australia. Clockwise from top left: *Hippocampus breviceps*; *Hippocampus subelongatus*; *Stigmatopora argus* and *Filicampus tigris*. Assay detections are indicated by the coloured dots detailed in the figure legend. Image credit: Dave Harasti-used with permission

brevicaudata). However, for the 16S_FishSyn_Short assay, this increase in detection rate may be due to its shorter amplicon length. As eDNA is released into the environment the degradation process begins (Bista et al., 2017), with short fragments of DNA degrading slower than larger fragments (Deagle, Eveson, & Jarman, 2006). Therefore, there may be a greater probability of detection using shorter amplicon assays relative to longer ones. While there is an increase in detection rates, previous studies have shown that assays targeting longer DNA fragments will selectively detect newly released eDNA (Jo et al., 2017; Wu et al., 2019), providing a more contemporary insight to fish community assemblage.

While they did not detect the largest number of species, the MiFish-U and 16S Fish assays detected 12 species that 16S_FishSyn_Short and 16S_FishSyn_Long did not. Evidently, each assay used has its own "blind spots" with overall taxonomic composition varying between assays. While primer mismatches (and base degeneracies) are common in PCR assays, the impact of these on taxa detections is difficult to evaluate in a set of environmental samples. By extrapolating the results, we have demonstrated that all fish metabarcoding assays will inadvertently omit a selection of the fish biota and that some groups (e.g., Syngnathidae) will be notable false negatives. Traditionally, studies have used one universal metabarcoding assay to estimate fish biodiversity (DiBattista et al., 2017; Stat et al., 2018). However, our data reiterates the importance of a multigene approach with multiple metabarcoding assays (Stat et al., 2017), albeit at an increased cost, as each assay has advantages and disadvantages related to diversity and taxonomic resolution based on the availability of reference sequences (Berry et al., 2017; Deagle, Jarman, Coissac, Pompanon, & Taberlet, 2014). Furthermore, the use of multiple metabarcoding assays can provide higher confidence levels when there are multiple (independent) assignments to the same organism (Stat et al., 2017).

With fish assemblages at each site displaying significant differences in the species detected, we demonstrated that eDNA surveys are ecologically informative over small spatial scales (~8.9 km average distance between sites). In accordance with other studies (Jeunen et al., 2019; Koziol et al., 2019; Murakami et al., 2019; O'Donnell et al., 2017; Stat et al., 2018; West et al., 2020), we suggest that eDNA signals dilute or degrade rapidly in aquatic ecosystems and are able to provide a contemporary snapshot of spatially distinct community assemblages. Furthermore, we identified a slight saltwater to freshwater gradient across the Perth metropolitan region and consistent with previous research, we suggest that eDNA signals are not impacted by localized oceanography (tides, currents and upwellings) as much as one might expect (Jeunen et al., 2019; O'Donnell et al., 2017).

The results of this study suggest that while the MiFish and 16S Fish assays might be capable of detecting the majority of teleost fish, they are unsuitable for detecting Syngnathidae species in complex multispecies environmental samples. Critical to the aims of this study, we successfully detected Syngnathidae species across a range of habitats in the Perth metropolitan region using the 16S_FishSyn_Short and 16S_FishSyn_Long assays. With Syngnathidae populations declining due to exploitation for the aquarium trade and habitat degradation (Luo et al., 2015; Martin-Smith & Vincent, 2006), eDNA provides a much-needed noninvasive method for monitoring threatened populations. Importantly, this study represents the first time a seahorse species has been detected using eDNA. Further optimizations (including the development of a Syngnathidae specific assay) are clearly needed as each of our assays (16S_FishSyn_Short and 16S_FishSyn_Long) detected different seahorse species in the water samples. The heterogeneity of eDNA in the environment can introduce a stochastic effect when sequencing sample and PCR replicates in which less abundant or smaller species may not be found in all replicates (Beentjes, Speksnijder, Schilthuizen, Hoogeveen, & Van Der Hoorn, 2019). While we took five sampling replicates, it has been suggested that as many as nine sample replicates are needed to obtain accurate biodiversity estimates from eDNA (Grey et al., 2018). This is further confounded by the fact that the relative biomass of seahorses to other fish biomass is likely extreme.

The frequency of detection for Syngnathidae species was low, less than 0.02% of the total reads. This indicates that the biomass of Syngnathidae DNA relative to other fish is low, possibly due to their: (a) low relative abundance, (b) low overall biomass, and/or (c) low DNA shedding rates. It is possible that Syngnathidae species may not have been detected in some samples as their DNA concentrations were below the detection threshold of the newly designed metabarcoding assays. Furthermore, not all eDNA sampling materials are ideal to detect all taxa and selection of eDNA material heavily influences assemblages derived from metabarcoding data (Koziol et al., 2019). In our empirical testing of bottom water versus surface water, Syngnathidae species were detected at a greater frequency in bottom water samples, with seahorse species only detected in bottom water. However, the low sample numbers are not sufficient to formally test this. We hypothesize that this difference could be due to the higher velocity associated with surface water dispersing and degrading DNA faster than bottom water where seahorses reside. This finding may hold importance for rare and cryptic species like Syngnathidae as their small relative biomass makes them challenging to detect with generic fish assays (Pikitch, 2018).

DNA metabarcoding relies heavily on the availability of high-quality sequences correctly identified by taxonomic experts for accurate species assignments. Current metabarcoding databases are incomplete as not all taxa have barcodes available, and this is further exacerbated for understudied organisms. The use of Western Australian 16S rRNA fish database provided an additional two species and one genus assignments, and increased taxonomic resolution for the 16S gene region. As the number of publicly available sequences grows, the probability of incorrect taxonomic assignment is reduced and taxonomic resolution is improved (Andújar, Arribas, Yu, Vogler, & Emerson, 2018; Somervuo et al., 2017). Curated databases with accurate sequences are of paramount importance to the growth of eDNA metabarcoding as a biodiversity survey tool and will increase the functionality and applicability of metabarcoding data (Andújar et al., 2018).

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The choice of barcoding region and assay can greatly affect species assignments and inferences on biodiversity (Cristescu, 2014; Zhang, Chain, Abbott, & Cristescu, 2018). Of the species that were not detected for the 12S gene region using the MiFish-U assay, 43.5% were missing from the NCBI database. In comparison, of the species that were not detected for the 16S gene region (using 16S Fish, 16S_FishSyn_Long and 16S_FishSyn_Short assays), an average of 15.9% of these were due to missing sequences from the NCBI and the Western Australian 16S rRNA fish database. This highlights the difference in taxonomic coverage and availability of fish sequences between the 12S and 16S mitochondrial gene region. The results of this study could have been affected by this difference through an underestimation of the number of species present, resulting in false negatives. While it is clear that these gaps in current databases will continue to impair the efficacy of eDNA metabarcoding for some time, the availability of reference sequences continues to grow and with it the likelihood of false negatives decreases. Our results emphasize the importance of making an informed choice on a suitable target gene region based on the availability of sequences for your target species.

5 | CONCLUSION

Through the development of 16S_FishSyn_Short and 16S_FishSyn_Long, we have successfully developed two novel fish eDNA metabarcoding assays capable of detecting Syngnathidae species, as well as a wide range of other fish taxa, in the marine environment. Consistent with previous research, our findings reiterate that no metabarcoding assay is "universal" (Stat et al., 2017), and that taxa of conservation importance like Syngnathidae may be missed from eDNA surveys due to a lack of suitable assays. The Syngnathidae family is a flagship group for conservation due to their captivating nature and the iconic status of several species (De Brauwer & Burton, 2018). However, with over 30% of species listed as data deficient, the difficulties associated with undertaking robust conservation assessments on this group are evident. Given the wide spread conservation concerns for these taxa (Vincent, Foster, & Koldewey, 2011), further eDNA work will focus on optimizing these Syngnathidae specific assays to develop an effective conservation toolkit for this family.

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CONFLICT OF INTEREST

None declared.

AUTHOR CONTRIBUTIONS

This study was designed by G.N., M.D.B., M.B., and J.D.D.; samples were collected by G.N. and M.D.B, samples were processed and molecular research was conducted by G.N., A.K., M.P., N.W., and K.W.; the reference database was created by J.D.D., A.K., and M.H.; metabarcoding assays were designed by K.W., A.K., and G.N.; statistical analysis was conducted by G.N. with advice from E.H., M.D.B, and M.B.; and all authors contributed to writing this paper.

DATA AVAILABILITY STATEMENT

Sequence data are available from https://www.ncbi.nlm.nih.gov/sra/?term=PRJNA565335, and the Western Australian 16S rRNA fish database sequences are available on NCBI under the accession MN473514 to MN473874.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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