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Improving the detection of rare native fish species in eDNA metabarcoding surveys

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Improving detection of rare species in eDNA surveys

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ABSTRACT

The presence of threatened/endangered species often strongly influences management and conservation decisions. Within the Murray-Darling Basin (MDB) (Australia) the presence of threatened native fish impacts the management and allocation of water resources. In New South Wales these decisions are currently based on traditional fisheries data and a predictive MaxEnt model. However, it is important to verify the model's predictive power given the implication it may have but this requires methods with a high detection sensitivity for rare species. Although the use of environmental DNA (eDNA) based monitoring, in particular eDNA metabarcoding, achieves a higher detection sensitivity compared to traditional methods, earlier surveys in the MDB have shown that the high abundant and invasive common carp (*Cyprinus carpio*) can reduce detection probabilites for rare species. Consequently, a PCR blocking primer designed to block the amplification of carp eDNA could increase the detection probabilities for rare native species while simultaniously reducing the required sampling effort and survey costs. While PCR blocking primers are often used in ancient DNA and dietary studies, no aquatic eDNA metabarcoding study to date has evaluated the potential benefits of using PCR blocking primers. A laboratory and field based pilot study was used to address this knowledge gap and assess the impact of a blocking primer, targeting cyprinid fishes (including carp), on the detection probabilities of native species and the minimum sampling effort required. The results showed that the inclusion of the blocking primer increased the detection probabilities for native species by 10 - 20 % and reduced the minimum required sampling effort by 25 - 50 %. These findings provide important insights into possible methods for optimizing eDNA metabarcoding surveys for the detection of rare aquatic species.

KEY WORDS

Environmental DNA, metabarcoding, blocking primer, detection sensitivity, fishes

INTRODUCTION

The Murray-Darling Basin (MDB) is Australia's largest river system covering approximately 14% of Australia's surface and spanning the states of New South Wales (NSW), Queensland, South Australia and Victoria, and the Australian Capital Territory (Koehn, 2015). Water allocation and policy throughout the MDB are strongly dependent on the presence of threatened native fish species (Koehn, 2015, Koehn and Lintermans, 2012). More detailed insights into the distribution of native fish species could thus improve water policies and assist species conservation.

Recently, the use of environmental DNA (eDNA) (i.e. DNA shed by organisms into the environment) analyses has proven to be a highly valuable tool for monitoring the presence/absence of rare and cryptic species (Ficetola et al., 2008, Jerde et al., 2011). Early studies utilized species-specific molecular approaches to detect the DNA of the taxa of interest and therefore infer their presence (Ficetola et al., 2008, Goldberg et al., 2011). However, this targeted approach quickly becomes expensive and time consuming when monitoring surveys focus on multiple taxa and therefore more universal monitoring approaches are favoured. In particular, the use of universal primers to amplify the eDNA from multiple target taxa combined with high throughput sequencing (HTS) technology (i.e. eDNA metabarcoding) is increasing in popularity (Jarman et al., 2018).

Although eDNA metabarcoding generally outperforms conventional monitoring techniques for fish (Hänfling et al., 2016, Shaw et al., 2016, Cilleros et al., 2019), recent studies have shown that the detection sensitivity for rare species is lower in eDNA metabarcoding surveys compared to a targeted approach (Bylemans et al., 2019, Harper et al., 2018). Studies have confirmed that the shear abundance of and/or the preferential primer annealing to the DNA of some taxa can hinder the detection of rare taxa (Vestheim and Jarman, 2008, Shehzad et al., 2012). While increased replication (at the sampling and amplification stage) can decrease the occurrence of false negatives (i.e. the failure to detect a species while it is present) (Ficetola et al., 2015), this approach will not affect the

actual detection probabilities and will increase labour and consumable costs. Increasing the detection probabilities for rare species could be achieved by selectively blocking the amplification of DNA that will be preferentially amplified. This can be done with PCR blocking primers, an approach often used to avoid the amplification of contaminant DNA (Boessenkool et al., 2012) or, in dietary studies, the DNA of the predator species (Vestheim and Jarman, 2008, Shehzad et al., 2012). However, no study to date has evaluated the feasibility of using PCR blocking primers to increase the detection probability of rare species in aquatic eDNA metabarcoding surveys.

Within NSW, government agencies rely on traditional fisheries data and a predictive MaxEnt model to determine the probability of occurrence for rare native species and guide water sharing rules. In particular, the presence of eight native priority species (i.e. *Ambassis agassizii*, *Bidyanus bidyanus*, *Maccullochella macquariensis*, *Maccullochella peelii*, *Macquaria australasica*, *Mogurnda adspersa*, *Nannoperca australis*, *Tandanus tandanus*), classified as threatened by state or commonwealth legislation, has important implications for water access rules and environmental water entitlements. However, verifying the predictive power of the model is crucial and eDNA metabarcoding surveys could be highly valuable for this. Previous surveys have shown that the relative high abundance of the invasive common carp (*Cyprinus carpio*), which in some cases can make up 70 - 90% of the fish biomass (Koehn, 2004, Lintermans, 2007), negatively influences the detection of rare species (Bylemans et al., 2018a). A pilot study was thus conducted with two main objectives. Firstly, the potential use of a carp blocking primer to increase the detection probabilities for rare native species was evaluated. Secondly, the impact of the blocking primer was assessed on the minimum sampling effort needed to assess the total native species diversity. The results of this pilot study are informative for future work within the NSW section of the MDB but also provides broader insights into potential methods for optimizing eDNA metabarcoding surveys for the detection of rare species.

MATERIALS AND METHODS

Development of a blocking primer

A cyprinid blocking primer (CBP) was developed to selective block the amplification of cyprinid DNA in environmental samples when using the AcMDB07 primers (Bylemans et al., 2018a). While the initial aim was to develop a carp-specific blocking primer, insufficient genetic variation was found in the regions directly adjacent to the primer binding regions to develop a highly species-specific blocking primer. Only the AcMDB07 primers were considered as other suitable metabarcoding primers either have a low taxonomic resolution (i.e. Teleo) or the regions adjacent to the primer binding regions were too invariable for the development of a blocking primer (i.e. MiFish-U) (Bylemans et al., 2018a, Valentini et al., 2016, Miya et al., 2015). Full details on the development of the CBP can be found in the Supporting Information.

Validation of the blocking primer

The performance of the CBP was first evaluated using a SYBR® Green Real-Time PCR assay to determine the effect of CBP concentrations on the amplification efficiency of carp DNA and DNA from three non-target species (*N. australis*, *M. australasica* and *Perca fluvatillis*). Full details on the PCR conditions are given in the Supporting Information and only briefly described below. Amplicons of the target gene region (i.e. 12S) were obtained and amplicon concentrations were standardised to 0.2 ng per PCR replicate. Four different concentrations of CBP were used in the PCR reactions (0, 0.2, 2 and 4 µM) and for each treatment (i.e. CBP concentrations by species combinations) six PCR replicates were performed. Real-Time PCR results (i.e. Cq-values) were imported into R version 3.5.2 (R Development Core Team, 2011) and ΔCq-values were calculated for individual PCR replicates (i.e. Cq-values obtained without the use of the CBP were subtracted from the Cq-values when different concentrations of CBP were used). Assuming a 100% amplification efficiency for CBP-

unbounded templates, the fold change (i.e. the proportional reduction in DNA amplification) can be calculated using the equation below.

$$Fold\ change = 2^{\Delta C_q}$$

Further validation of the CBP was performed using eDNA samples collected from two sites within the main channel of the Murrumbidgee River. Both sites were deemed highly suitable for further validation of the CBP as they are known to be occupied by multiple native and endangered species and have a relatively high biomass of invasive carp (Table 1). A total of twelve 1 L water samples were collected from each site and processed following protocols outlined in Bylemans et al. (2018b) (Supporting Information). Appropriate cleaning processes were used, and negative controls were included during sampling, filtering and eDNA extractions (Supporting Information). For all eDNA samples 1:10 dilutions were prepared to minimise the impact of PCR inhibitors. Negative control samples were screened for the presence of fish eDNA using Real-Time PCR and if amplification was observed, negative controls were included in the HTS library construction step. Sequencing libraries were constructed using a one-step PCR amplification with and without the CBP (Bylemans et al., 2018a) (Supporting Information). Triplicate PCR reactions were performed, and amplicon pools were constructed through two pooling steps. Two PCR clean-up and left-handed size selection steps were used during pooling and the final library was send to the Ramaciotti Centre for Genomics (University of New South Wales, Australia) for paired-end sequencing on an Illumina MiSeq platform using the v2 2x250bp sequencing kit.

Data analyses

The raw sequence data was filtered using a bio-informatics pipeline based on the OBITOOLS software (Boyer et al., 2016) following the general workflow as described in De Barba et al. (2014) and Bylemans et al. (2018a) (Supporting Information). Further filtering of the metabarcoding data

was performed using R version 3.5.2. Fish sequences present in the negative control samples were used to set a minimal threshold value for the sequence counts in the eDNA samples (i.e. sequence counts below the threshold value were discarded). Finally, the data was checked for ambiguous taxonomic assignments and other sources of errors (e.g. chimeric sequences) on a case-by-case basis considering the relative sequence abundance, the taxonomic assignments and the barcode sequences.

Statistical analyses were conducted in R version 3.5.2 using the packages tidyverse (Wickham, 2017), vegan (Oksanen et al., 2007) and iNEXT (Hsieh et al., 2016). Firstly, the overall species richness detected at both sites, with and without the use of the CBP, was evaluated to assess the overall performance of the metabarcoding workflow. Detection probabilities for all native fish species were calculated (i.e. proportion of samples per site returning a positive detection) with and without the use of the CBP. A paired sample t-test was used to evaluate whether the detection probabilities of the native species differed with or without the use of the CBP. Finally, the metabarcoding data for the native species was transformed to presence/absence data before constructing species accumulation curves using the iNEXT function to evaluate the minimum sampling replication needed to accurately assess the native fish biodiversity.

RESULTS

Development of a carp blocking primer

A CBP was designed that contains a 3 base-pair (bp) long section at the 5'-end that overlaps with the reverse metabarcoding primer (i.e. AcMDB07-R). A C3 spacer at the 3'-end of the CBP will prevent elongation during PCR amplification. The CBP will thus prevent the annealing of the AcMDB07-R primer to cyprinid DNA and thus reduce PCR amplification. Full details of the AcMDB07 metabarcoding primers and the CBP are given in Table 2.

Validation of the blocking primer

When using a 2 µM concentration of CBP the proportional reduction in the amplification of carp DNA was close to zero, indicating an almost complete blocking of PCR amplification (Figure 1). For non-target species, a modest reduction (25 %) in the amplification efficiency was observed when 0.2 and 2 µM of the CBP was added to the PCR reaction (Figure 1). At the highest CBP concentration (i.e. 4 µM), the amplification of non-target DNA is reduced by approximately 75% relative to the controls indicating that high CBP concentrations may have adverse effects on the detection of non-cyprinid species. Based on these results, a 2 µM concentration of the CBP was used for all subsequent analyses.

After the bio-informatics filtering of the raw HTS data, the total reads assigned to fish species ranged from 87,681 to 359,099, with a mean of 177,157 reads for each uniquely labelled sample. Further details on the quality of the run and the reads discarded during the bio-informatics processing can be found in the Supporting Information.

The species richness detected at each site (Figure 2) shows that the total number of native species detected with or without the CBP does not differ. While the number of invasive species detected with and without the CBP is the same for the Buckingbong site, the use of the CBP decreases the number of invasive species detected at Casuarina Sands. This decreased detection of invasive species was due to a positive detection of rainbow trout (*Oncorhynchus mykiss*) in a single sample when the CBP was not included in the PCR amplification (Supporting Information).

When evaluating the impact of the CBP on the detection probabilities for all native species, the paired sample t-test revealed a significant difference between the samples analysed with and without the CBP ($P< 0.05$) (Figure 3). In most cases the inclusion of the CBP increased detection probabilities for native species by approximately 10 to 20 % (Figure 3). Furthermore, the species accumulation curves show that fewer samples are required to accurately assess the native fish

biodiversity in both sites when the CBP was used (Figure 3). While the use of the CBP in the Buckingbong site halved the minimum number of samples needed to detect 95 % of the native species, at the Casuarina Sands site the minimum number of samples needed was reduced by 25 % when the CBP was included in the PCR amplification (Figure 3).

DISCUSSION

The inclusion of the CBP increased the detection probabilities for native fish species. While previous research has indicated that eDNA metabarcoding surveys may suffer from false negative detections (Bylemans et al., 2019, Harper et al., 2018), the results obtained here show that by selectively blocking the amplification of eDNA from highly abundant species the risk of false negatives can be reduced. These results are congruent with ancient DNA and dietary studies which have shown that the proportion of reads assigned to rare taxa can be increased by blocking the amplification of DNA from unwanted taxa (Boessenkool et al., 2012, Shehzad et al., 2012).

Previous studies have highlighted the need for sufficient replication at the sampling and PCR amplification stage to mitigate the risk of false negatives and accurately characterise the species community (Ficetola et al., 2015, Bylemans et al., 2018b, Cilleros et al., 2019). However, an increase in replication will also increase the workload and survey costs. The results obtained from this pilot study show that selectively blocking the amplification of highly abundant eDNA to be a valid alternative strategy. The results also highlight that relative template concentrations in mixed samples causes primer-template competition during PCR amplification and this may be a major limiting step in the eDNA metabarcoding workflow. While these findings are not necessarily new, within the metabarcoding literature considerable attention has been payed to the effects of primer-template bp mismatches (Elbrecht and Leese, 2017, Piñol et al., 2015, Bylemans et al., 2018a) but the effects of different template starting concentrations, and the interactive effects between the two, remains poorly understood (Kalle et al., 2014, Kanagawa, 2003). Nonetheless a thorough understanding of primer-

template dynamics in multi-template PCR reactions is needed to determine the most optimal strategies to reduce false-negatives in DNA metabarcoding studies. For example, in samples with low evenness in DNA templates, the highly abundant templates may consistently mask the detection of rare ones and thus increasing PCR replication may not be the most suitable and/or economical approach.

In practice, the use of a PCR blocking primer in aquatic eDNA metabarcoding surveys requires some prior information or assumption about the most dominant species in the survey area. While this can be obtained when systems have been monitored before (i.e. with traditional surveys or eDNA-based surveys), the use of PCR blocking primers may be more difficult to implement in poorly studied systems. A two-step analyses approach could be used in poorly studied systems where the first round of analyses follows a standard eDNA metabarcoding approach, while in the second round the DNA amplification of highly abundant species can be selectively blocked. Although this will also increase turnaround times and costs, in some cases (e.g. samples with low evenness or when variation between PCR replicates is low) it may be a more suitable approach to verify and improve the detection of rare taxa. Another important practical consideration is that blocking primers cannot be developed for all metabarcoding primers. For fish, the regions directly adjacent to the MiFish-U primer binding regions have low interspecies variability which makes it challenging to design suitable blocking primers.

Finally, while the inclusion of the CBP generally increased the detection probability of rare taxa the results also showed that in some instances the use of the blocking primer decreased the detection probabilities (Figure 3). This was the case for *Macquaria ambigua* at the Buckingbong site and *B. bidyanus* and *Oncorhynchus mykiss* at the Casuarina Sands site (Supporting Information). Both detections of *M. ambigua* and *O. mykiss* at the respective sampling sites were derived from a single sample which may arise from the stochastic nature of sampling or PCR amplification. The *B. bidyanus* detections at the Casuarina Sands site originated from three samples analysed without the CBP. In all

three samples low numbers of *B. bidyanus* reads were observed thus suggesting low DNA concentrations. Stochastic effects during sampling or PCR amplification could have attributed to the observed results and thus the *B. bidyanus* represent real detections even though the presence of this species in the Casuarina Sands site was deemed unlikely (Table 1). Alternatively, the observations could be explained by low amounts of cross-contamination between the Buckingbong and Casuarina Sands samples during laboratory protocols. However, appropriate measures during laboratory workflows and the bio-informatics filtering were taken to eliminate the impact of contaminants.

CONCLUSION

In conclusion, the results presented here have shown that selectively blocking the amplification of DNA from highly abundant species can improve the detection of rare taxa while also reducing required sampling replication needed in eDNA metabarcoding surveys. While both increased replication and the selective blocking of highly abundant DNA can be suitable strategies to reduce false negative detections, more research is needed to understand primer-template dynamics in mixed DNA samples. Such research will provide critical information about the most critical steps that should be considered when aiming to reduce false negatives. Overall, the most suitable approach for limiting false negatives is likely to vary on a case by case basis and a multitude of factors (e.g. time, costs, prior information, etc.) will need to be carefully considered.

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DATA ACCESSIBILITY

The summarized eDNA detection data are present in the Supporting Information (Table S1 & S2).

AUTHOR CONTRIBUTION

DMG, TH and JB conceived the idea; JR and JB designed the study and performed the necessary field work; JR performed all laboratory analyses; JR and JB conducted the data analyses; JR and JB wrote the manuscript with significant contributions from DMG and TM. All authors gave final approval for the publication of the manuscript.

TABLES

Table 1. Details of the sampling sites within the main river channel of the Murrumbidgee river samples for the validation of the cyprinid blocking primer. Site details are given along with the predicted presence of the eight native priority species.

Sampling site	**Predicted presence of the eight priority species**	
(Latitude; Longitude)	**Common name**	**Scientific name**
Casuarina Sands	Trout Cod	*Maccullochella macquariensis*†
(-35.3190389; 148.9581944)	Murray Cod	*Maccullochella peelii*
	Macquarie Perch	*Macquaria australasica*
	Silver Perch	*Bidyanus bidyanus*†
Buckingbong	Trout Cod	*Maccullochella macquariensis*
(-34.803504; 146.616136)	Murray Cod	*Maccullochella peelii*
	Silver Perch	*Bidyanus bidyanus*
	Eel Tailed Catfish	*Tandanus tandanus*‡

† Species have not been recorded in State Government surveys since 2008 (Bylemans et al., 2018b), ‡ Presence unlikely but possible. Casuarina sands species data obtained from Lintermans (2002) and Bylemans et al. (2018b). Buckingbong species data obtained from Gilligan (2005) (Trout Cod and Silver Perch) and M. Duncan. Pers. Comm (2019) (Murray Cod and Eel Tailed Catfish).

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Table 2. Details of the primers and the cyprinid blocking primer (CBP) used in further metabarcoding analysis. Primers were developed previously (Bylemans et al., 2018a) while the CBP was developed in the current study. The overlapping region between the CBP and reverse metabarcoding primer is underlined.

Primer ID	Sequence (5'-3')	Amplicon
AcMDB07-F	GACCCTATGGAGCTTTAGAC	*ca.* 320 bp
AcMDB07-R	GTACACTTACCATGTTACGACTT	
AcMDB07-CYPR-RB	CTTGCCTCCCCTTGTCAGTGCTG-c3	

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FIGURE LEGENDS

Figure 1. The calculated fold change as a function of the concentration of the Cyprinid Blocking Primer (CBP) for four species (horizontal panels) and two primer pairs (vertical panels).

Figure 1. The overall species richness detected at the two sampling sites with and without the use of the Cyprinid Blocking Primer (CBP). Results are shown for both native and invasive species.

Figure 2. The results of the paired sample t-test used to evaluate whether the detection probabilities for the native fish species changed with and without the use of Cyprinid Blocking Primer (CBP) (upper panel). The species accumulation curves for each site using the data obtained with and without the use of the CBP are given in the lower panel with the dashed vertical lines indicating the minimum number of samples needed to detect 95% of the expected species richness (i.e. Chao2 estimate).