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INVASION NOTE

Something in the water: biosecurity monitoring of ornamental fish imports using environmental DNA

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Abstract The international trade in ornamental aquatic organisms represents an important vector in the spread of invasive species worldwide, but the accurate identification of imported organisms as part of a biosecurity surveillance program offers an opportunity to mitigate potential problems. Species level identification is historically conducted visually, and more recently, with the use of DNA barcoding. However, new diagnostic methods targeting extracellular environmental DNA (eDNA) can offer advantages over these approaches, being non-destructive and potentially more sensitive at low population densities of target organisms (e.g. in mixed consignments). Despite their recent introduction, techniques utilising eDNA are quickly becoming recognised as an important tool for invasion biologists and ecosystem managers. Here, we present a model for the development of a biosecurity protocol for ornamental fish identification using degraded eDNA molecules in water. We demonstrate how a DNA barcode reference library can be mined for informative short-length markers, and report repeatable and accurate detection at low densities of the target species. This study represents a framework for biosecurity agencies to develop eDNA procedures as an innovative management technique for routine surveillance of high risk imports. Future up-scaling of the method will open up prospects for long term monitoring of entire quarantine facilities for a variety of harmful species.

Keywords Biosecurity · DNA barcoding · eDNA · Ornamental fish trade · Quarantine · Sliding window

Introduction

International trade, and in particular the aquarium industry, is a significant vector for non-indigenous fishes, invertebrates and associated pathogens (Duggan 2010; Chang et al. 2008; Derraik and Phillips 2010). With over one billion fishes traded annually throughout the world (Whittington and Chong 2007), and upwards of 5,000 species represented (Hensen et al. 2010), there is a requirement for effective biosecurity monitoring of these threats. Quarantine procedures can play a substantial role in preventing possible incursions (Meyerson and Reaser 2002). As discussed by Collins et al. (2012, and references

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therein), different countries address the problem via one of two strategies: a blacklist of taxa prohibited, or a whitelist of taxa permitted. However, enforcing these lists at the border is problematic, due to difficulties associated with visual identification of fishes to species level (McDowall 2004), and the presence of incidental hitchhiking organisms entering the supply chain via wholesale facilities or bycatch (Duggan 2010).

DNA barcoding has been presented as a standardised solution to some of the difficulties in identifying the large number of species from the ornamental fish trade (Collins et al. 2012). This approach requires tissue sampling the imported animals. However, destructively sampling entire individuals may not be possible if the fish are valuable, or only a single example is available. Alternatively, fin clips or swabs can be taken, but these may leave the fish susceptible to infections by breaking the skin or removing the protective mucous layer (Le Vin et al. 2011). Perhaps more importantly, however, tissue sampling requires that the "unknown" or "query" individual has *a priori* been acknowledged as such. Therefore, the tissue sampling approach may not be sufficiently sensitive to detect small numbers of similar looking hitchhikers that may be difficult to observe.

A potential solution lies with the use of extracellular environmental DNA (eDNA), which can now be accessed from a diverse range of substrates, including water (Darling and Mahon 2011). In aquatic ecosystems, assessment of presence and abundance can now be made, an approach allowing the detection and monitoring of invasive species (Ficetola et al. 2008; Jerde et al. 2011), rare and secretive species (Goldberg et al. 2011), or community composition as a whole (Thomsen et al. 2012). In terms of invasive species monitoring, Ficetola et al. (2008) reliably detected the presence of invasive bullfrogs in both controlled conditions and in natural ponds, while Jerde et al. (2011) delimited invasion fronts of two Asian carp species. Despite the relatively recent introduction of the technique, eDNA analyses are quickly becoming recognised as an important tool for invasion biologists and ecosystem managers (Darling and Mahon 2011).

Using eDNA, we have the ability to detect presence of a target species among other species in an ornamental fish shipment, which is important in terms of assessing fish abundance and composition in mixed consignments. Because water will to some degree hold a "molecular memory" of the species present in it, eDNA protocols can therefore also track the historical presence of a species in a water sample. This may be of benefit if a particular high-risk taxon has been in recent close contact with an otherwise low-risk species at a wholesaler or transshipper. This would perhaps justify added precautions to be taken in terms of disease risk and quarantine.

Given the instability of DNA in aqueous environments (Dejean et al. 2011), and the need for short-length molecular markers for degraded DNA studies, an important but rarely explored aspect in eDNA study design is the choice of region that will still discriminate among species (Boyer et al. 2012). In this respect, we will evaluate an approach using a standard length DNA barcode reference library (651 base pairs) to mine markers suitable for specimen identification from water using eDNA. Ideally, the most informative regions should be chosen, but to some degree the choice is limited by the availability of suitable priming sites. Sliding window analyses can therefore be used as a tool to evaluate variability through a gene alignment and find informative regions flanked by less variable priming locations, or, for species specific applications, to locate diagnostic sites for fluorescent probe design.

In this study, we provide a standardised protocol to further develop the eDNA method for biosecurity. Specifically, we: (a) describe an objective technique for selecting a species-specific marker; (b) test this marker for specificity to a target taxon; (c) in a simulated international transport scenario, demonstrate that short-length eDNA amplicons can be reliably retrieved from water at low densities of the target species; and that (d) target species can continue to be detected when mixed with closely-related non-target species.

Materials and methods

Target species

The chosen target species was the model organism *Danio rerio* (zebrafish or zebra danio). This species of cyprinid fish is easy to manipulate in the laboratory, and is also popular in the ornamental aquarium trade.

Primer design using sliding windows

The cytochrome *c* oxidase subunit I (COI) DNA barcode reference library of cyprinid fishes—as

generated by Collins et al. (2012)—was chosen as the base for mining a short-length species-specific eDNA marker. A total of 139 individuals from 19 *Danio* species were taken from the dataset. Sequences shorter than the full 651 base pair (bp) alignment were removed from the COI alignment, resulting in 127 individuals (Online Resources 1). The alignment was analysed in R version 2.15 (R Development Core Team 2012), using the *slidingWindow* function of the DNA barcoding package SPIDER (Brown et al. 2012). As described in Boyer et al. (2012), *slidingWindow* takes a fixed length of DNA (in this case 100 bp), and from the first base of the full alignment, moves along at one base intervals to generate 551 windows of width 100 bp. For each window, a series of calculations are made on the information content or discriminatory power (Boyer et al. 2012). For this analysis we used the number of species diagnostic sites (*nucDiag* function) to highlight potentially useful locations for species-specific primers. An R script outlining the sliding window process is presented in Online Resources 1. Primers were then designed manually, and checked for Tm (melting temperature) and GC base content using PRIMER3 with default parameters (Rozen and Skaletsky 2000). The primer pair designed here for the *D. rerio* specific eDNA fragment is presented in Table 1.

Primer specificity: in vitro PCR

PCR amplification of the eDNA primer pair was tested against 46 previously extracted tissue samples from 25 species of *Danio* and representatives of closely related genera (Online Resources 2). Tissue extractions had been stored in elution buffer at −20 °C, and were between 18 and 38 months old (for protocol see Collins et al. 2012). Approximately two specimens of each species were tested, comprising different haplotypes where possible. Although it is unlikely that these samples would have degraded since extraction, testing for primer specificity relies on a negative PCR result (no amplification = poor primer fit). Therefore, to be confident in the result, a control for DNA degradation was carried out: full length DNA barcodes were amplified in parallel on the same tissue extractions using universal fish primers. Optimised PCR conditions are described in Online Resources 3.

Primer specificity: in silico PCR

To test if organisms other than the immediately related ones (i.e. those tested in the in vitro experiment) are likely to amplify with the eDNA primers, a search was made using the program MFEPRIMER (Qu et al. 2009). MFEPRIMER is able to evaluate the "specificity of PCR primers based on multiple factors, including sequence similarity, stability at the 3′ end of the primer, melting temperature, GC content and number of binding sites between the primer and DNA templates" (Qu et al. 2009). All COI sequences were downloaded from GenBank (date: 02/02/2012) under the search term "COI" (total 810,305 sequences). A local installation of MFEPRIMER was run under both default settings (word size 11, and *e* value 1,000), and more stringent settings (word size 7, and *e* value 10,000).

eDNA detection: experimental treatments

Environmental DNA experiments were carried out in 20 L containers, each with an air supply. Fishes were transferred from stock tanks to the containers using sterilised equipment, and were left in the container overnight in a dark room for 16 h to simulate an international transit scenario. Three 15 mL samples (total from each replication 45 mL) were collected from the surface in three 50 mL conical centrifuge tubes.

Two density treatments were employed using the target species *Danio rerio*: (a) one fish in 4 L of water (∼0.24 g fish per litre); and (b) one fish in 12 L of water (∼0.08 g fish per litre). Each treatment was

Table 1 Primers generated in this study for species-specific detection of *Danio rerio* using environmental mitochondrial DNA from the COI locus

Primer name	Direction	Start/end position	Primer sequence 5′–3′	Length (bp)	Tm (°C)	GC (%)
eDR3fwd	Forward	6,456	ATCATAAAGACATTGGCACCCTG	23	62.28	43.48
eDR3rev	Reverse	6,551	GCTAAGTTCAGCTCGGATTAAG	22	57.52	45.45

Resulting amplicon length 95 bp. The start/end positions refers to the location of the amplicon in reference to the *D. rerio* mitochondrial genome (GenBank accession NC_002333)

repeated four times in sets of four and included one negative control container on each occasion (total 12 repetitions with fish, and four without fish); i.e. for every three replicates, each container was in turn used as a negative control (no fish added). Average fish mass was estimated by placing 25 fishes in a water-filled beaker on a digital balance, and a mean taken (=0.95 g).

To test effectiveness for detecting species in mixed samples, a further treatment (c) was carried out in 4 L of water with a single *D. rerio* as a "hitchhiker" among 19 individuals of the closely related species *D.* aff. *kyathit* (1:20 dilution factor). Replications were carried out as described above in four sets of four, with each negative control comprising 20 of the non-target species (*D.* aff. *kyathit*) present (i.e. target species absent).

Isolation of the eDNA was carried out following a sodium acetate ethanol precipitation method outlined in Online Resources 3, together with details of PCR conditions and gel electrophoresis. Three PCRs were carried out on each extraction, with a positive identification comprising a single band at the expected length ($\sim$100bp) in at least one of the PCRs. From each of the three treatments, four positive PCR products were chosen at random to be bidirectionally Sanger sequenced (protocol as Collins et al. 2012). See Online Resources 3 for further methodological information.

Fish experiments and DNA extractions were carried out in dedicated rooms free of PCR product contamination. A schematic outline of experimental procedure for a single replication of water sampling from one container is shown in Online Resources 3. All equipment was sterilised after each experiment for a minimum of 3 h with 1.25 % sodium hypochlorite solution (Kemp and Smith 2005), comprising one part 5 % bleach solution to three parts water. As both fishes and DNA molecules are sensitive to chlorine, containers were rinsed repeatedly with tap water and treated with SEACHEM PRIME.

eDNA detection: operational testing

An additional experiment was also carried out in an operational biosecurity context, with water samples taken from a shipment of the target species (*Danio rerio*) at an MPI Biosecurity New Zealand transitional (quarantine) facility. Therefore, there were fewer experimental controls when compared with the treatments outlined above. Specifically: (a) eDNA isolation was carried out by different personnel in a separate laboratory, thereby testing that the protocol was robust; (b) the imported fish were of an unknown haplotype, thereby testing primer fit; and (c) PCR may be inhibited by either the high concentration of fish metabolites in the fouled shipping water, or the chemicals added by shippers to remove these metabolites. The fish were first identified visually by officials, and multiple water samples were then taken from the shipment bag (3 $\times$ 15 mL as previously). See Online Resources 3 for further methodological details.

Results

Sliding window analysis and primer design

With the *slidingWindow* function set to 100 bp, the highest frequency of diagnostic nucleotides for *Danio rerio* was within the first 100 bases of the COI barcode marker (Fig. 1). Primer design was therefore targeted in this area (see "Materials and methods"). The resulting amplicon comprised a total of 95 base pairs, and started at position 6,456 through position 6,551 of the *D. rerio* mitochondrial genome (GenBank accession NC_002333).

Primer specificity

For the in vitro tests of primer specificity, the eDNA primers amplified three individuals of the 46 specimens tested (Online Resources 2); these all corresponded to specimens identified as *D. rerio*. No species tested other than *D. rerio* were amplified. For the overall assessment of DNA quality from the stored extractions, all amplified full length DNA barcodes (Online Resources 2).

The in silico tests of primer specificity using the MFEPRIMER program under default settings made three matches from the local database (COI sequences from GenBank) that could potentially produce a PCR product; all three of these were from the target species. Under the more stringent *e* value and word size settings, two additional matches were found. These were from a South American bird (*Jacamerops*

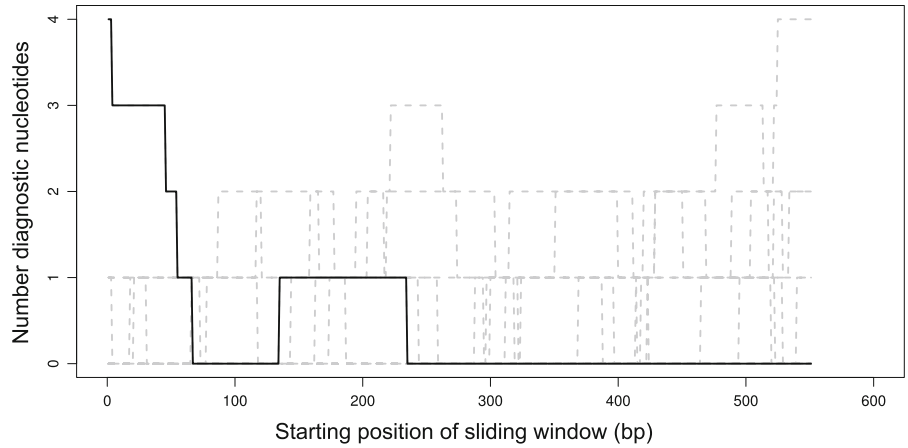

Fig. 1 Diagnostic nucleotide sites for a 100 base pair sliding window across the COI barcode marker for the genus *Danio*. *Solid line* is number of discriminating sites specific to *Danio rerio*, while *dashed lines* are discriminating sites specific to individual non-target *Danio* species ($n = 18$)

aureus), and a bacterium (*Bacillus pseudofirmus*); the latter had an amplicon length of 2,304 bp.

eDNA detection: experimental treatments

For density treatment (a)—single fish in 4 L of water—a total of 48 PCRs were carried out, with three PCR reactions for each replicate; 12 of these PCRs were the negative experimental control (no fish in container). All PCR reactions (three per replicate) were positive for *D. rerio* (amplicon present of expected length). None of the negative experimental controls showed a band of expected length. Results for density treatment (b)—single fish in 12 L of water—and mixed species treatment (c) were identical to treatment (a). PCR products from all treatments and replicates were of consistent, strong intensity under electrophoresis; Online Resources 2 shows a sample agarose gel image. The subsample of PCR products for which sequences were obtained showed clean chromatograms identical to that of the *D. rerio* mitochondrial genome (GenBank accession NC_002333).

eDNA detection: operational testing

The two sets of water samples taken from a shipment bag of *D. rerio* at the quarantine facility both tested positive for this species in all six PCR reactions. The sequenced PCR product was, again, unambiguously *D. rerio*. The extraction and PCR controls were both negative.

Discussion

Primer design and specificity

The sliding window analysis was found to be a useful tool for identifying regions in DNA alignments for the development of species specific primers. The primers designed here were specific to the target for all in vitro PCR reactions of closely related species, and the positive tissue-sample controls showed that stored DNA extractions had not deteriorated below a point where a standard DNA barcode could be amplified. As measured by the in silico experiment using MFEPRIMER, there appears to be a low likelihood of non-target amplification, with a small number of hits for well corresponding sequences. Of course, this conclusion is entirely dependent on the breadth of sequence data present in GenBank, and bias here cannot therefore be entirely avoided.

eDNA detection and sources of error

In both experimental and operational experiments using 45 mL of aquarium water, it is shown here that eDNA can be isolated at varying fish densities. These densities correspond to those well below the densities at which fishes are typically exported (Cole et al. 1999; Ploeg et al. 2012); amplification was successful here at fish densities of both 0.08 and 0.24 g/L, while exports from Singapore are frequently above 34 g/L, and up to 264 g/L (Ploeg et al. 2012). In terms of

transit times, these are often reported to be longer than the 16 h used here, with over 30 h total transit time from Singapore to Europe (Ploeg et al. 2012). This, together with results showing the detection of the target species at a 1:20 factor to mixed non-targets, suggests the technique could therefore be sufficiently sensitive to detect low numbers of individuals within mixed shipments of fishes. Additionally, the scaling down of this procedure from 45 mL into a 1.7 mL microcentrifuge tube was found to be possible (results not shown), and further testing of this could be important for high throughput work.

Due to the sensitive nature of PCR reactions using large numbers of cycles, routine eDNA monitoring for biosecurity requires a rigorous assay design to ensure confidence in the results (Willerslev and Cooper 2005; Darling and Mahon 2011). Tests must be robust to errors, and these errors need to be well understood if the method is to be endorsed for use in management situations where there are political, financial, and legal stakes (Darling and Mahon 2011). Furthermore, in practice, it is also critical to distinguish between false positive and false negative errors caused by either the process or the method used (Fig. 1 of Darling and Mahon 2011).

In the context of ornamental fish biosecurity, a false negative error would result when the target species is present in the shipment, but is not detected due to an inadequate or insufficiently sensitive methodology. For example, DNA purification may not remove PCR inhibitors, primers may fail to amplify an unexpected haplotype, or the water sample volume too small to reliably recover degraded eDNA at low concentrations (Darling and Mahon 2011). A false positive error would occur when the target species is detected, but that organism is not present in the shipment. This may occur due to laboratory contamination, poor primer specificity (non-target species amplified), or the historical presence of the target organism in the water sample (i.e. target no longer present). This latter scenario is a potential issue when dealing with the importation of aquarium species from wholesalers using multi-tank water recirculating systems; a consignment of non-target species may be shipped in water containing DNA molecules of a target species. While this may appear a problem in terms of false positive error, it can also be of considerable benefit for biosecurity; knowing whether a shipment has been recently associated with a known pathogen vector would be useful in terms of disease risk management. As part of a routine surveillance procedure, however, any positive PCR result could be carefully followed up during quarantine with a visual or tissue-based molecular identification approach, or alternatively, the sensitivity of the test could be reduced (e.g. smaller water volumes).

Summary

The results here support the usefulness of eDNA as a biosecurity tool for ornamental fishes, and represents a framework and protocol for developing the procedure further. Environmental DNA surveys offer advantages over traditional techniques such as visual examination and barcoding from tissue samples, as they are non-destructive and potentially more sensitive at low population densities of target organisms.

Single taxon assays using standard or quantitative PCR are especially suitable as a routine test for a small number of blacklisted species or genera. However, even when combined as a multiplex PCR reaction, this system is limited in its taxonomic scope. Fortunately, up-scaling of the method opens up prospects for long term, multi-species monitoring of entire quarantine facilities or ornamental fish retailers using next-generation meta-barcoding technologies (Andersen et al. 2012), or mini-barcode microarray systems (Hajibabaei et al. 2007).

The availability of large volumes of COI data from databases such as the Barcode of Life Database (BOLD), can allow mining of useful new eDNA markers for single species or groups of species. As part of the standardised DNA barcode system, these mini-barcodes remain compatible with the voucher specimens and supplementary data associated with those records, adding confidence to identifications. However, in order to fully capitalise upon next generation sequencing technologies, it is required that current DNA barcode reference libraries for ornamental fishes be expanded beyond those currently available. Some high-risk fish groups have already been targeted; for example, 74 of the 82 cyprinid fish species permitted into New Zealand have already been DNA barcoded (Collins et al. 2012), but more are required. In terms of determining biosecurity priorities among the huge variety of potentially risky ornamental species, objective assessment of potential

targets can now be achieved using tools such as FISK, the Fish Invasiveness Scoring Kit (Copp et al. 2009).

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