Parasite detection in the ornamental fish trade using environmental DNA

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Effective border control relies on stringent biosecurity protocols to detect and prevent introductions of exotic pests and diseases. Detection of pathogens and parasites in the live ornamental fish trade using environmental DNA (eDNA) techniques has the potential to improve current biosecurity practices. We examined water samples from 11 target consignments (cyprinids susceptible to Dactylogyrus spp. infections) and seven non-target fish consignments (non-cyprinids, not susceptible to Dactylogyrus spp. infections) imported from Southeast Asia to Australia for the presence of eDNA from five Dactylogyrus species (Monogenea: Dactylogyridae). A four-step predictive framework was used to predict putative positive and putative negative detections from quantitative PCR assays. Both target and non-target consignments were positive for Dactylogyrus spp. eDNA as confirmed by Sanger sequencing. Positive detections for Dactylogyrus spp. eDNA in non-target fish consignments demonstrates the possibility of source water contamination, limiting the applicability of eDNA screening methods at border control. This study suggests that screening for parasite eDNA within ornamental fish consignments should be tested during pre-export quarantine periods to avoid false positive detections at border control. Lastly, the proposed predictive framework has a broad utility for minimizing false positive and false negative eDNA detections of aquatic organisms.

The ornamental fish trade is a known route of exotic pathogen translocations globally. Parasites and their infected hosts have been co-introduced to non-native environments with detrimental effects on biodiversity, ecosystems, industries, and dependent local communities. To minimize pathogen translocation through the ornamental fish trade, governments can establish quarantine measures based on scientific risk analyses that consider the origin and history of fish stocks, parasite life cycles, host susceptibility to infection, risk of transmission to native species, and the reliability of detection methods. Australia for example, has stringent mandatory pre-export quarantine requirements, biosecurity protocols at border control, and post arrival mandatory quarantine requirements following strict biosecurity import risk assessments of ornamental fish imports. Despite current biosecurity protocols, recent surveys of ornamental fish species imported to Australia have shown that a high diversity of parasites were not detected during inspection at border control, highlighting the need for more detection sensitivity. Considering the limitation of gross visual inspection under current biosecurity protocols it is important to explore new and complimentary methods to increase biosecurity rigor and the possible integration of molecular genetic techniques.

Environmental DNA (eDNA) refers to the DNA that is naturally shed by organisms, such as through epidermal sloughing, metabolic waste excretions or post-mortem decay, into their local environment. In the case of microscopic parasites, life stages like eggs, spores, cysts, larvae, juveniles and adults can be present in the water column, in sediment, or in extracellular DNA disassociated from host organisms. As such, parasite genomic (gDNA) and nucleic (nDNA) can be captured with eDNA samples, extracted, and screened for target species using standard molecular genetic techniques like quantitative real-time polymerase chain reaction (qPCR).

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Environmental DNA could enable species-level detection and monitoring in aquatic parasitology with important benefits to human health, animal welfare, freshwater fisheries, coastal aquaculture, conservation, and ecosystem health. Indeed, captured and extracted eDNA from water samples has been shown to accurately detect pathogenic trematodes infecting wild amphibians and to monitor parasites infecting farmed and wild fish species. Environmental DNA was recently proposed to be a non-destructive and sensitive detection tool for biosecurity, and was used to determine the presence of ornamental fish species at low densities within high risk mixed imports. Screening water used to import ornamental fish consignments for the presence of parasites has the potential for biosecurity monitoring advancement; however, there are no studies to date that have specifically tested this utility of eDNA.

False positive and false negative errors are commonly encountered in qPCR analyses. From a biosecurity perspective, misinterpreting qPCR data could lead to pathogen-free consignments being considered hazards during quarantine inspection (i.e., false positive error), or high-risk pathogens going undetected in infected consignments (i.e., false negative error). As such, preventative measures must be developed to ensure accurate interpretation of qPCR data and reduce the possibility of false positive and negative results.

The aim of this study was to determine if eDNA screening by qPCR is a plausible detection tool for biosecurity. A four-step predictive framework was designed to minimize the possibility of false positive and false negative qPCR detections for the presence or absence of five ectoparasitic monogenean flukes (Dactylogyrus anchoratus, D. formosus, D. intermedius, D. vastator and D. ostraviensis) previously detected by necropsies infecting ornamental cyprinid fishes (Carassius auratus and Pettia conchonius) imported from Southeast Asia to Australia.

**Methods**

**Dactylogyrus spp. eDNA collection.** All water samples analysed for the presence of eDNA from Dactylogyrus species in this study were collected during a cross-sectional survey for the presence of nationally listed aquatic pathogens associated with at least one ornamental fish host. Briefly, 37 ornamental fish consignments representing 11 farmed freshwater and seven marine wild caught fish species were imported from Southeast Asia to Australia in 2015 following Australian Biosecurity Import Conditions (BICON) and subjected to Australian quarantine protocols, which involved gross visual inspection and clearance by Australian Quarantine Services. A 'consignment' of fish was defined as a unique fish species within a shipment of fish, identified by an invoice containing details of the numbers and species of fish, date of shipment, origin and destination, accompanied by health certification. Following release from quarantine inspection, all consignments were transported by road to an Approved Arrangement Site (AA Site) at the University of Sydney (Camden, Australia).

Freshwater consignments arrived at the AA Site in either one large plastic bag or several medium plastic bags, containing 40 to 200 individuals depending on species and size. Each plastic bag contained approximately 1–5 L of freshwater and was sealed with either rubber bands or metal clasps. All consignments were housed inside large Styrofoam boxes during transit (12–48 hours including export, delivery, inspection, and release to the importer) before water samples were collected from each consignment and preserved. Negative controls (distilled water) were collected prior to collecting triplicate 15 mL samples from each fish consignment. To minimize the risk of eDNA cross contamination, each 15 mL replicate was collected from all plastic bags holding each consignment using a new disposable 20 mL sterile glass pipette attached to an automatic pipette controller (EasyPet, Eppendorf). Water samples were dispensed directly into individual pre-labelled DNA-free 50 mL centrifuge tubes, each with 33.5 mL absolute ethanol and 1.5 mL 3 M sodium acetate for preservation and then stored at room temperature. Following water sample collection, 30 fish from each consignment were randomly selected, euthanized, and examined for the presence of monogenean parasites by necropsy, as described in a separate study. In brief, all 30 fish were sequentially surveyed for external parasites by an experienced parasitologist using a compound microscope to carefully examine gill samples from each fish for the presence or absence of parasites. A sample size of 30 fish per consignment was selected to achieve a minimum detection prevalence of 10% with 95% confidence limits determined by using exact binomial approximation. As such, samples where no parasites were detected by necropsy were considered to have an apparent prevalence of 0%, with a 95% confidence interval (CI) of 0–11.4%, assuming a perfect test. Environmental DNA was extracted using cetyl trimethylammonium bromide (CTAB), which included phenol-chloroform isolation and terminal isopropanol precipitation. All DNA was resuspended in 60 µL 1x Tris-EDTA (TE) buffer and stored at −20 °C until screening for Dactylogyrus spp. eDNA by qPCR. Animal ethics, method and sampling approval was obtained from the University of Sydney Animal Ethics Committee (approval number: 720) and all methods were performed in accordance with guidelines and regulations of the University of Sydney Animal Ethics Committee.

**Design of species-specific Dactylogyrus primers and assay validation.** Novel species-specific oligonucleotide primers were designed to detect and discriminate between five Dactylogyrus species (Monogenea: Capsalidae): Dactylogyrus anchoratus (Dujardin, 1845), D. formosus Kulwiec, 1927, D. intermedius Wegener, 1909, D. ostraviensis Rehulka, 1988, and D. vastator Nybelin, 1924. All five Dactylogyrus spp. are highly specific to cyprinid fish hosts and other helminths given its low intraspecific yet high interspecific variability. Each Dactylogyrus-specific primer was designed to target the ITS1 region that contained the most mismatches (>1) between target and all non-target Dactylogyrus species (Table 1). To achieve this, previously accessioned Dactylogyrus spp. ITS1 nucleotide sequences were downloaded from GenBank (NCBI) and aligned using ClustalW (www.genome.jp/tools/clustalw, version 1.81).

All qPCR assays were tested for specificity in silico using the National Center for Biotechnology Information (NCBI) Primer BLAST, Amplify4 (engels.genetics.wisc.edu/amplify), and Amplifx 1.7.0 (Nicolas Jullien;
Table 1. Primers for *Dactylogyrus* spp. ITS1 eDNA assay. The efficiency, $R^2$, and limit of detection for each quantitative PCR assay is provided. Primer cross-reactivity tests are provided in Support Information 1.

| Parasite species | Primer (bp) | Annealing (°C) | Primer sequence (5′-3′) | qPCR efficiency (%) | $R^2$ | Limit of detection (ng/μL) |
|------------------|-------------|----------------|-------------------------|---------------------|-------|--------------------------|
| *Dactylogyrus anchoratus* | D. anchoratus F | 185 | 60 | 5′-GCCATCCCTGGAGAATATGCACCA-3′ | 75.12 | 0.981 | 0.00065 |
| | D. anchoratus R | | | 5′-GAGTTCAGGCGGCCCACAT-3′ | | | |
| *Dactylogyrus formosus* | D. formosus F | 184 | 65 | 5′-CTCATCCCTGGAGAATATGCACCA-3′ | 119.55 | 0.984 | 0.0079 |
| | D. formosus R | | | 5′-AAGTTCAGGCGGCCCACAT-3′ | | | |
| *Dactylogyrus ostraviensis* | D. ostraviensis F | 120 | 65 | 5′-TCTGTCGATGACGCTGG-3′ | 97.3 | 0.98 | 0.00092 |
| | D. ostraviensis R | | | 5′-CACATACTGGACGCTGCCC-3′ | | | |
| *Dactylogyrus vastator* | D. vastator F | 210 | 60 | 5′-GGGCGAGCTGACCTAGCCA-3′ | 98.99 | 0.95 | 0.0009 |
| | D. vastator R | | | 5′-AGACTGCGACGACGTGACTAAC-3′ | | | |
| *Dactylogyrus intermedius* | D. intermedius F | 210 | 60 | 5′-TCAGATCCTGACCCCTATCACTAC-3′ | 104.6 | 0.982 | 1.32E-07 |
| | D. intermedius R | | | 5′-TGCCGACACGACGTGTTA-3′ | | | |


### Stepwise criteria for eDNA detection and tests for *Dactylogyrus* spp.

A four-step conservative framework was developed to minimise the risk of false positive and false negative results in qPCR assay analysis. These criteria were selected considering the need to accurately determine absence from disease in biosecurity 29 and future applications of Tm analysis to ensure accurate and reliable detection. For each qPCR assay the Tm of each amplicon was compared to the mean Tm of the corresponding species-specific gDNA, which was calculated from all technical qPCR replicates across the entire standard curve ($± 99.7%$ CI). The absolute difference between the mean Tm of the species-specific gDNA standard curve and each individual qPCR technical replicate amplicon within a corresponding species-specific assay ($ΔTm$) was calculated by subtracting $Tm_{sp}$ of each technical replicate amplicon from the mean $Tm_{sp}$ curve of the corresponding species-specific gDNA standard. Calculated $|ΔTm|$ values were then used to categorise each putative positive detection (i.e., amplicon) into one of three confidence levels: *Cl 1* = high (amplicon expected to be positive for *Dactylogyrus* spp. detection), *Cl 2* = medium (amplicon suspected to be positive for *Dactylogyrus* spp. detection), and *Cl 3* = low (amplicon predicted to not be positive for *Dactylogyrus* spp. detection, i.e., false positive) (Fig. 1).

Amplicons were categorized as *Cl 1* if: (1) amplification curves crossed the common threshold fluorescence within 40-cycles (Criterion 1.1, Fig. 1), (2) $Tm$ values were within 99.7% CI of the corresponding species-specific mean gDNA standard $Tm_{ss}$ (Criterion 2: Cl 1, Fig. 1), and (3) agarose gel visualization confirmed length to match that observed and expected for corresponding species-specific gDNA standard (Criterion 3, Fig. 1). Amplicons were categorized as *Cl 2* if they matched *Cl 1* criteria (see above) but exhibited a $|ΔTm|$ outside 99.7% CI and $≤ 1°$C from mean $Tm$ of corresponding species-specific standards (Criterion 2: Cl 2, Fig. 1). Amplicons were categorized as *Cl 3* if they matched *Cl 1* criteria but exhibited $|ΔTm|$ outside 99.7% CI and $> 1°$C from mean $Tm$ of corresponding standards (Criterion 2: Cl 3, Fig. 1). Putative positive *Cl 1*, *Cl 2*, and *Cl 3* amplicons were Sanger sequenced (Australian Genome Research Facility, Brisbane) for *Dactylogyrus* spp. level confirmation (NCBI BLAST; Criterion 4, Fig. 1). If any given *Dactylogyrus* spp. eDNA assay had $≥ 2$ putative positive amplicons categorized as *Cl 1* or *Cl 2* then two representatives for each *Cl* were chosen for Sanger sequencing (one with lowest and one with highest $|ΔTm|$ value), otherwise one or both putative positive amplicons were sequenced. If any *Dactylogyrus* spp. eDNA assay had $≥ 2$ putative positive amplicons categorized as *Cl 3* then the
amplicons with the lowest and highest $|\Delta T_m|$ values (i.e., most and least likely to be confirmed as positive detections) were sequenced, otherwise both putative positive amplicons were sequenced.

Amplicons were considered to be putative false negative detections if no amplification curves were produced or failed to cross the common fluorescence threshold within 40 cycles (Criterion 1.2) but exhibited $|\Delta T_m|$ values within 99.7% CI of mean $T_m$ of corresponding species-specific standards (false negative, Fig. 1). Amplicons categorized as putative false negatives were re-amplified by qPCR to determine if a $|\Delta T_m|$ value within 99.7% CI of mean $T_m$ of corresponding species-specific standards and expected amplicon length were produced when amplified using 1$\mu$L of PCR product from initial amplification. False negative amplicons were re-amplified using six replicate 20$\mu$L qPCRs containing 1$\mu$L of post-PCR product, 1$\mu$L of each PCR primer (400 nM), 10$\mu$L PowerUP® SYBR GreenER qPCR Master Mix (Life Technologies, Australia) and 8$\mu$L MilliQ® water, and were run under the same cycling conditions described above. Any amplicons produced from qPCR re-amplification that met Criteria 1, 2, and 3 (see above; Fig. 1) was Sanger sequenced for confirmation.

If an entire assay did not produce any amplicons that crossed common fluorescence threshold within 40 cycles (Criterion 1.2, Fig. 1) and no amplicons exhibited a discernible $T_m$ then the entire assay was repeated. An assay was considered negative if neither initial or subsequent qPCR runs produced amplicons that crossed common fluorescence threshold within 40 cycles (Criterion 1.2, Fig. 1) and neither initial or subsequent qPCR runs produced amplicons with detectable $T_m$ (Criterion 2, Fig. 1).

Species-specific qPCR assays were used to test extracted DNA in water samples from target and non-target fish consignments for the presence of Dactylogyrus spp. eDNA (Fig. 2). Import data were considered 'target' or 'non-target' fish consignments based on published records of infection for any of the Dactylogyrus spp. targeted in this study ($n = 5$). Based on this criteria, seven goldfish (Carassius auratus (Linnaeus, 1758)) consignments were considered targets for D. anchoratus, D. formosus, D. intermedius, and D. vaster whereas four rosibarb (Pethia conchonius (Hamilton, 1822)) consignments were considered targets for D. ostraviensis (Fig. 2). Based on the same criteria, one guppy (Poecilia reticulata, Peters 1859), one pearl gourami (Trichogaster lalius (Bleeker, 1852), one three-spot gourami (Trichogaster trichopterus (Pallas, 1770)), one green swordtail (Xiphophorus maculatus (Günther, 1866)) consignments were considered non-target hosts for all five Dactylogyrus spp. species. All target and non-target host fish consignments were screened for the presence of eDNA from all five Dactylogyrus species using species-specific qPCR assays (Fig. 2) followed by assessment of each produced amplicon based on selection criteria described above (Fig. 1).

Results

Positive Dactylogyrus spp. eDNA detection in target fish consignments. Dactylogyrus spp. eDNA was detected in all consignments where Dactylogyrus spp. were detected by standard necropsies. Specifically, eDNA from D. formosus and D. vaster was detected in water samples from all C. auratus consignments, and eDNA from D. anchoratus and D. intermedius was detected in all consignments except for consignments 4 and 6, respectively (Fig. 2). Dactylogyrus anchoratus was detected by both approaches (eDNA and necropsy) in consignments 6 and 7 while neither approach detected parasites in consignment 4. Dactylogyrus ostraviensis eDNA was detected in all target P. conchonius consignments, while necropsies did not detect D. ostraviensis in consignment 12 (Fig. 2). Dactylogyrus spp. eDNA was detected in five C. auratus and one P. conchonius consignments considered to have Dactylogyrus spp. apparent prevalence of 0% (95% CI 0–11.4%) by necropsy10 (Fig. 2). No eDNA was detected in negative controls.

Positive Dactylogyrus spp. eDNA detections in non-target fish consignments. A total of 39 amplicons produced across all 58 qPCR tests of non-target fish consignments were confirmed positive for Dactylogyrus spp. eDNA (Fig. 2). Dactylogyrus formosus, D. intermedius, and D. vaster eDNA was detected
Table 1. Taxonomic distribution of Dactylogyrus spp. recordable from importation of fish consignments into the EU.

| Fish species     | Exporter | Dactylogyrus anchoratus | Dactylogyrus formosus | Dactylogyrus intermedius | Dactylogyrus vastator | Dactylogyrus ostraviensis |
|------------------|----------|-------------------------|-----------------------|-------------------------|-----------------------|-------------------------|
|                  | Necropy  | eDNA                    | Necropy               | eDNA                    | Necropy               | eDNA                    |
| Cometogaster auratus | Singapore 2 | -                     | 4/6*                  | -                       | 4/6*                  | -                       |
| Cometogaster auratus | Singapore 2 | -                     | 0/12                 | -                       | 6/6*                  | -                       |
| Cometogaster auratus | Thailand 1 | -                     | 4/6*                  | -                       | 6/6*                  | -                       |
| Cometogaster auratus | Thailand 1 | 26.6 (12–46)           | 4/6                   | 6.7 (0.82–22)           | 5/6                   | 13.3 (3–27)             |
| Cometogaster auratus | Thailand 1 | 3.3 (0.1–17.2)         | 4/6                   | 43.3 (25–63)            | 0/12                  | 40 (20–59)              |
| Cometogaster auratus | Malaysia 1 | -                     | 1/6*                  | -                       | 6/6*                  | -                       |
| Cometogaster auratus | Malaysia 1 | 5/6*                   | 6.6 (0.82–22)         | 3/3                     | 3.3 (0.1–17.2)        | 3/3                     |
| Pterhos cochlus | Singapore 2 | -                     | 1/6                   | -                       | 2/2                   | -                       |
| Pterhos cochlus | Thailand 1 | -                     | 3/6                   | -                       | 1/6                   | -                       |
| Pterhos cochlus | Thailand 1 | -                     | 1/6                   | -                       | 1/6                   | -                       |
| Pterhos cochlus | Malaysia 1 | -                     | 1/6                   | -                       | 1/6                   | -                       |
| Mesocotyle reticulata | Sri Lanka | -                     | 1/6                   | -                       | -                     | -                       |
| Trichapodus leperi | Sri Lanka | -                     | -                     | -                       | -                     | -                       |
| Trichapodus trichapodus | Thailand 1 | -                     | -                     | -                       | -                     | -                       |
| Xiphophorus affinis | Sri Lanka | -                     | -                     | -                       | -                     | -                       |
| Xiphophorus hulotensi | Turkey 1 | -                     | 1/6                   | -                       | 4/6                   | -                       |
| Xiphophorus hulotensi | Singapore 2 | -                     | -                     | -                       | -                     | -                       |
| Xiphophorus hulotensi | Singapore 2 | -                     | -                     | -                       | -                     | -                       |

Figure 2. Comparison between necropsies and environmental DNA (eDNA) detection of Dactylogyrus species in imported ornamental fish consignments. Detections by necropsy presented as mean apparent prevalence % (95% Confidence Interval, CI)10 and eDNA detections as confirmed positive amplicons/total number of amplicons. Grey areas indicate qPCR assays of target fish consignments, and asterisks (*) indicate consignments where Dactylogyrus spp. were not detected by necropsies but were detected by eDNA assays. Negative symbols (−) indicate that no parasites were detected in a total of 30 fish and had an apparent prevalence = 0% (95% CI = 0–11.4%)10, and that no parasite eDNA was detected from a total of six eDNA sample replicates.

in P. conchonius consignment 13 (Singapore 2; Fig. 2). Dactylogyrus intermedius and D. ostraviensis eDNA was detected in X. maculatus consignment 24 (Singapore 2, Fig. 2) while D. vastator and D. intermedius eDNA was detected in X. maculatus consignment 23 (Thailand 1; Fig. 2). Similarly, D. ostraviensis eDNA was detected in C. auratus consignments 3 and 4 as well as X. maculatus consignment 24 (Singapore 2; Fig. 2). Lastly, D. formosus, D. intermedius, D. vastator, and D. ostraviensis eDNA was detected by qPCR in P. reticulata consignment 17, T. leeri consignment 18, and X. maculatus consignment 25 (Sri Lanka; Fig. 2). No Dactylogyrus spp. were reported in non-target fish consignments by necropsies10.

Accuracy of predictive framework. All amplicons categorized as high confidence of Dactylogyrus detection (CL 1) from all Dactylogyrus spp. qPCR assays were confirmed positive by Sanger sequencing (Fig. 1 Criterion 4). All amplicons categorized as moderate confidence (CL 2) from D. anchoratus, D. formosus, and D. intermedius qPCR assays were also confirmed positive by Sanger sequencing (Fig. 1 Criterion 4). Of the amplicons categorized as CL 2 from D. ostraviensis and D. vastator qPCR assays, 80% and 87.5% (n = 4/5 and 7/8) were confirmed positive by Sanger sequencing, respectively. These two CL 2 amplicons were unable to be confirmed as positive detections due to poor sequencing quality (i.e., not due to non-target amplification; see Fig. 3D for D. ostraviensis and Fig. 4 for D. vastator).

No low confidence (CL 3) categorized amplicons from D. anchoratus, D. formosus, D. intermedius, or D. ostraviensis qPCR assays were confirmed positive by Sanger sequencing. However, 81.25% (n = 13/16) of CL 3 categorized amplicons from D. vastator qPCR assays were confirmed positive by Sanger sequencing (Fig. 1, Criterion 4). One D. vastator qPCR assay amplicon from T. tricopterus consignment 14 was initially considered a putative false negative (Fig. 1 Criterion 2) but was subsequently categorized as CL 1 following qPCR reamplification (Fig. 1) and confirmed positive by Sanger sequencing (Fig. 1, Criterion 4, Fig. 4 “amplicon 19_4”). All other putative false negative amplicons produced during Dactylogyrus spp. eDNA assays were confirmed negative following the selective framework (Fig. 1, Support Information 2).

Amplicon sequence confirmation. All confirmed positive D. anchoratus amplicons were 100% homologous to D. anchoratus ITS1 GenBank sequences (AJ564111, AJ490161, MF356241, KY859795, MF662103, MF356243, and MF356242). All confirmed positive D. formosus amplicons were 100% homologous to D. formosus ITS1 GenBank sequences (AJ564135, MF356239, KM526669, KX369215, and KC876018). All confirmed positive D. intermedius amplicons were 100% homologous to D. intermedius ITS1 GenBank sequences (KX369220, MF356236, MF356244, KJ854364, MF356237, and MF356240). All confirmed positive D. ostraviensis amplicons were 100% homologous to D. ostraviensis ITS1 GenBank sequences (MF356250 and MF356249; which are the only two sequences available)10.

Confirmed positive D. vastator amplicons, unlike all other Dactylogyrus spp. amplicons, separated into two distinct groups (Fig. 4). Dactylogyrus vastator Group 1 amplicons exhibited an average Tm ± SD of 86.64 °C ± 0.59 with average |∆Tm| being ±0.6 °C away from Tm of gDNA standards (|∆Tm|); Fig. 4), while amplicons in Group 2 exhibited an average Tm ± SD of 85.37 °C ± 0.47 with average |∆Tm| being ±1.97 °C away from Tm of gDNA standards (Fig. 4). The six confirmed positive D. vastator amplicons that fell within the 99.7% CI of D. vastator gDNA standards (Group 1) were 98–100% homologous to the following D. vastator ITS1 GenBank sequences: MF356235 (Thailand), KY207446 (Croatia), AJ564159 (Czech Republic), MF805686 (Iran), MF356246 (Thailand), KY201104 (Italy), and KY201092 (Bosnia and Herzegovina). The 11 positive D. vastator amplicons that fell outside the 99.7% CI of the same D. vastator gDNA standards (Group 2) were 96–100% homologous to...
the following D. vastator ITS1 GenBank sequences: KX369223 (China), MF356247 (Thailand), KY201103 (Czech Republic), and KM487695 (China). Groups 1 and 2 D. vastator amplicons differed by a total of 16 fixed nucleotide differences (Support Information 3).

Figure 3. Absolute difference in melting temperature (|ΔTm|) between sequenced amplicons derived from environmental DNA assays for Dactylogyrus anchoratus (A), Dactylogyrus formosus (B), Dactylogyrus intermedius (C) and Dactylogyrus ostraviensis (D) and their corresponding genomic DNA standards. Grey and black bars in Panels A–D represent confirmed positive and confirmed negative amplicons, respectively. Horizontal dotted lines in Panels A–D represent the upper 99.7% Confidence interval for Tm of species-specific standards. **Forward and reverse sequences were low in quality; however, a 72 bp fragment of consensus alignment was found to be 100% similar to Cyprinus carpio GenBank sequence LN599613 (i.e. considered as confirmed negative).

Figure 4. Absolute difference in melting temperature (|ΔTm|) between Dactylogyrus vastator amplicons derived from environmental DNA (eDNA) assays and genomic DNA (gDNA) standards confirmed by Sanger sequencing. Grey and black bars represent confirmed positive and confirmed negative amplicons, respectively. Horizontal dotted lines represent the upper 99.7% CI for Tm of serially diluted D. vastator gDNA standard. Group 1 amplicons had 1–2 base pair differences between sequences obtained compared to D. vastator gDNA standard, while Group 2 amplicons had 2–18 base pair differences between sequences obtained compared to D. vastator gDNA. Asterisk (*): consensus sequence could not be determined for this amplicon because reverse sequence failed; however, forward sequence had 93.8% similarity to Contracecum sp. [GenBank accession KM463761] and 91% similarity to Contracecum rudolphii Hartwich, 1964 [GenBank accession JQ071409] and thus this amplicon was considered as a confirmed negative detection. ClustalW alignment of all D. vastator ITS1 amplicon sequences provided in Support Information 3.
Discussion

The developed qPCR assays detected Dactylogyrus spp. eDNA in all consignments where necropsies detected Dactylogyrus spp.24. Species-specific qPCR assays were able to detect Dactylogyrus spp. eDNA in six target fish consignments, where necropsies considered Dactylogyrus spp. to have an apparent prevalence of 0% (95% CI 0–11.4; Fig. 2). As such, qPCR-based eDNA detection had higher surveillance sensitivity than necropsies, detecting Dactylogyrus spp. DNA in triplicate 15 mL water samples and confirming amplicons by Sanger sequencing.

However, D. intermedius, which was reported to infect C. auratus in consignment 6 by necropsy30 was not detected by eDNA screening in any qPCR technical replicates (n = 12; Fig. 2). Consequently, this was the only false negative eDNA detection observed in this study (1/90 tests; Fig. 2). It is possible that D. intermedius present in consignment 6 were genetically distinct from D. intermedius infecting consignments 5, 7, 8 and 9 (Fig. 2). The possibility of unique ITS1 genotypes in D. intermedius is supported by sequenced data of D. vastator, which displayed two ITS1 genotypes observed across screened goldfish consignments (Fig. 4; Support Information 3). Unlike the D. vastator assay, the D. intermedius assay appears to target an ITS1 region that is sufficiently hyper-variable to prevent primer binding27,34,35; however, this was unknown at the time of assay development due to limited nucleotide sequence information available for D. intermedius populations. Such a lack of comprehensive nucleotide sequence information has also limited other molecular genetic studies aimed at investigating parasite diversity.26,27 As such, successful implementation of the four-step predictive framework relied on the comprehensiveness of species-specific gDNA standards, suggesting \(|\Delta T_m|\) analysis requires careful interpretation given the inherent dependence on sequence homology between amplicons and standards for targeted gene(s) that may or may not be known. This study highlights the need for comprehensive nucleotide sequence data and robust corresponding morphological taxonomy to ensure accuracy of designed qPCR assays and corresponding standards for \(|\Delta T_m|\) analyses.

A total of 39 amplicons from non-target fish consignments were confirmed positive for Dactylogyrus spp. eDNA (Fig. 2). Considering that all Dactylogyrus spp. in this study are highly specific to cyprinid species24,25,33, positive detections in water samples from non-target consignments suggest that detected eDNA was not present due to active shedding from live infesting Dactylogyrus parasites. This interpretation is further supported by the absence of infection records for the selected Dactylogyrus specimens in non-target host fish species24,25 and non-detection by necropsies (Fig. 2). Dactylogyrus spp. are ectoparasites that occur naturally in southeast Asia3,8 and their environmental stages (i.e., eggs and oncomiracidia) could be present in recirculating aquaculture systems, raceways, or ponds used to rear freshwater species by exporting companies. As such, it is possible that exporters could have used a water source contaminated with Dactylogyrus spp. environmental life stages or degraded eDNA to transport exported fish consignments. If exporters do not use clean (e.g., filtered or UV treated) water to export ornamental fish consignments, then the accuracy and interpretability of eDNA assays at border control is limited, given that their applicability would depend greatly in differentiating between live infections and dead or inactive environmental parasite stages in the water column. Furthermore, considering that Australian quarantine officers have limited time to process imported consignments, eDNA-based detection by qPCR may not be applicable or reliable at border control using \(T_m\) analysis to carefully interpret qPCR results within an acceptable timeframe and biosecurity standard.

Screening water samples for parasite eDNA by qPCR could be a valuable detection method during pre-export quarantine periods. Current risk analyses from the Australian Government Department of Agriculture and Water Resources aim to ensure off-shore biosecurity in exporting countries24 by enforcing strict regulations and health requirements prior to export6. For example, all imported goldfish consignments must be certified free of infection from gill flukes Dactylogyrus extensus and D. vastator prior to export6. Both species are reported to cause significant economic losses in Asian cyprinid aquaculture3,40, and could pose significant risks to Australian aquarium shops selling cyprinids if live parasite infections go undetected during quarantine6.

Detection of eDNA by qPCR assays could be conducted on ornamental fish consignments during the mandatory quarantine period prior to export to support mandatory pre-export health certifications6. For instance, qPCR assays could be developed to assess the origin of parasite eDNA based on DNA decay rates by targeting various DNA fragment lengths41–43. Abundant long DNA fragments would indicate active shedding from live parasites while abundant short DNA fragments would indicate degrading DNA in the absence of live, shedding organisms41–43. Similarly, qPCR assays could also assess cellular activity by targeting environmental RNA (eRNA)12,43,44. Environmental RNA is indicative of active gene transcription and is proportionally less abundant in dormant stages than in metabolically active stages12,43. Given that RNA is less able to persist extracellularly and degrades quickly in dead or sloughed-off cells12, detection of eRNA by qPCR could be employed to determine the presence of metabolically active parasites infecting fish ready for export. Future research should consider designing qPCR assays to differentiate between active parasite infections and dead or non-active parasite stages and the applicability of eDNA detection during pre-export quarantine periods.

In conclusion, this first attempt at applying eDNA to ornamental fish parasite biosecurity highlights both the utility of incorporating molecular methods into biosecurity protocols as well as the limitations that need to be addressed if future applications and full integration are to be successful. We present a novel and comprehensive four-step predictive framework (Fig. 1) for the accurate interpretation of species-specific eDNA data and reduce false positive and false negative detections generated by Sybr-based qPCR assays. The interpretability and reliability of eDNA detection at border control specifically is limited; however, eDNA screening could prove highly valuable if implemented following pre-export quarantine periods. Further research needs to address limitations encountered in this study and test the viability of eDNA-based detection methods in other stages of quarantine and biosecurity surveillance.
Data Availability
Data for this study can be accessed as: Trujillo Gonzalez, A. (2018). Parasite detection in the ornamental fish trade using environmental DNA. James Cook University. [Data Files] https://doi.org/10.25903/5b90c1897397a.

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**Author Contributions**

Alejandro Trujillo-González wrote the main manuscript text, prepared all figures, extracted all DNA associated to the study, designed all qPCR assays and ran all qPCR tests and analyses. Dr Richard C. Edmunds contributed to the editing process of the manuscript text, designed the primers used in all qPCR assays and provided advice during qPCR testing and analysis. Dr Joy A. Becker contributed to the editing process of the manuscript text, sample collection, and was the primary researcher of the FRDC grant 2014/001: Aquatic Animal Health Subprogram: Strategic approaches to identifying pathogens of quarantine concern associated with the importation of ornamental fish. This grant funded all materials and equipment needed for sampling and qPCR analysis. Dr Kate S. Hutson is the primary supervisor of Alejandro Trujillo-González, contributed to the editing process of the manuscript text, sample collection, provided advice during qPCR analysis and was the secondary researcher of the FRDC grant 2014/001.

**Additional Information**

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