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Application of environmental DNA analysis to inform invasive fish eradication operations

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Abstract Environmental DNA (eDNA) detection of non-native species has considerable potential to inform management decisions, including identifying the need for population control and/or eradication. An invasive species of European concern is the Asian cyprinid fish, topmouth gudgeon (*Pseudorasbora parva*). Here, eDNA analyses were applied at a fishery site in southern England to inform operations aiming to eradicate *P. parva*, which had only ever been observed in one of the venue's seven unconnected angling ponds. Eradication of *P. parva* was initially attempted by repeated depletion of the population using fish traps (crayfish traps fitted with 5 mm mesh netting) and the introduction of native predators over a four-year period. The very low number of *P. parva* captured following these eradication efforts suggested a possible population crash. Conventional PCR analysis of water samples using species-specific primers was applied to all seven ponds to confirm that *P. parva* was present in only one pond, that the eradication attempt had indeed failed and that the species' distribution in the pond appeared to be restricted to three bankside locations. The continued presence of *P. parva* at these locations was confirmed by subsequent trapping. Water samples from an adjacent, unconnected stream were also analysed using the eDNA methodology but no DNA of *P. parva* was detected. The results suggest that further management action to eradicate *P. parva* be focused on the pond shown to contain the isolated *P. parva* population and thereby eliminate the risk of further dispersal. This study is the first to apply eDNA analysis to assess the efficacy of an eradication attempt and to provide evidence that the species was unlikely to be present in the other ponds, thus reducing the resources needed to control the species.

Introduction

Surveys based on the detection of environmental DNA (eDNA) are increasingly used to detect the presence of a broad range of taxonomic groups in aquatic environments, with particular applications to species of conservation concern and non-native species (Rees et al. 2014; Thomsen and Willerslev 2015). This is because eDNA-based surveys; which collect DNA shed by an organism via urine, faeces, mucus and epidermal cells into the water; tend to have greater power to detect elusive and/or rare organisms than conventional sampling approaches, e.g. bluegill sunfish (*Lepomis macrochirus*) (Takahara et al. 2013). This increased effectiveness, combined with relatively low financial costs and reduced impact on the environment, demonstrates that eDNA methodologies have high potential for enhancing the management of invasive fish species (Rees et al. 2014, Bylemans et al. 2016). Applications so far have included distribution assessments (Takahara et al. 2013; Keskin 2014), monitoring surveys on invasion fronts (Jerde et al. 2013; Adrian-Kalchhauser and Burkhardt-Holm 2016), and the evaluation of population eradication attempts (Dunker et al. 2016).

Eradication of potentially harmful non-native species is considered a key component of invasive species management, particularly in rapid response scenarios (UK Defra 2008; Britton et al. 2011a; Genovesi et al. 2015). Attempts to eradicate non-native fish species often involve application of a piscicide, such as rotenone (Allen et al. 2006; Britton et al. 2008), even though this practice can have substantial impacts on non-target fauna (e.g. Finlayson et al. 2010; Billman et al. 2011). In some circumstances, such as isolated water bodies, it may be possible to eradicate a fish species through a drain-down and liming of the water body (Britton et al. 2008). Other options for controlling invasive fish populations include repeated cropping by netting, trapping or electric fishing, and biological control by stocking predators (Britton et al. 2008).

Topmouth gudgeon (*Pseudorasbora parva*), a native species in eastern Asia, is one of the most invasive freshwater fish species in Europe, having spread across most of the continent within decades of its accidental introduction to Romania in the 1960s as a contaminant of Asian carp consignments (Gozlan et al. 2010). It arrived in England by this introduction vector in the mid-1980s (Gozlan et al. 2002). Such is the threat posed by *P. parva*, in particular its role as a healthy host of the rosette agent *Spherotecum destruens* (Gozlan et al. 2005), that it is the target of a national eradication campaign, which aims to remove all 23 known UK populations by the end of 2017 (UK EA 2014; GBNNSS 2015). *Pseudorasbora parva* is one of just two fish species currently listed as being of European Union concern under Regulation (EU) No. 1143/2014, requiring EU member states to implement management and control measures (European Union 2014). Methods which have been successfully used to eradicate local topmouth gudgeon populations include rotenone treatments and repeated removals (Copp et al. 2007). Also, there are instances elsewhere in Europe where *P. parva* have established a population in a water body, persisted for a short period (<10 years) and then disappeared entirely (Copp et al. 2007). This suggests that the species may be susceptible to recruitment failure and local extirpation where their population numbers are dramatically reduced by either natural or human assisted means.

To facilitate this management programme, an attempt to eradicate a *P. parva* population from a pond on a commercial recreational angling venue in southern England was undertaken between 2011 and 2016 using depletion and biocontrol methods. Given the requirement of such eradication attempts to undergo thorough post-operation evaluations to measure their efficacy (Britton et al. 2011a), the aim of this study was to demonstrate the potential use of eDNA analysis as a complement to conventional sampling methodologies for assessing the efficacy of fish eradication attempts. Our specific objectives were to: (1) develop a statistically-robust eDNA sampling protocol for evaluating the *P. parva* eradication attempt;

(2) assess the efficacy of the eradication attempt using conventional and eDNA methods; and (3) determine whether or not *P. parva* was likely, based on eDNA analysis results, to be present in any other water bodies at the site.

Materials and methods

Primer design and testing

Species-specific primers for *P. parva* were designed to amplify a 350 base-pair region of the mitochondrial gene encoding cytochrome *c* oxidase subunit 1 (COI): forward primer (5′-3) CCTCTTCCGGAGTAGAGGCT and reverse primer (5′-3) TAGGATTGGGTCTCCTCCCC (Davison et al. 2016). Primer specificity was tested *in silico* against sequences of all UK freshwater fishes, using NCBI Primer-BLAST (http://www.ncbi.nlm.nih.gov/tools/primer-blast/). The primers were also tested experimentally in conventional PCRs against DNA extracts (DNeasy Blood and Tissue Kit, Qiagen, Hilden, Germany) from fish species from the same family (Cyprinidae) which are likely to occur at the study site: common carp (*Cyprinus carpio*), common bream (*Abramis brama*), roach (*Rutilus rutilus*) and rudd (*Scardinius erythrophthalmus*). Conventional PCRs were conducted using 0.1 ng of genomic DNA and none of the triplicate PCRs showed amplification for any of these species.

Testing of primer efficiency and optimisation of the PCR protocol was undertaken using DNA extracted from dorsal muscle tissue samples of *P. parva*. These tests showed that the primers reliably amplified *P. parva* DNA at a quantity of 1.5×10^{-2} ng. The ability of the primers to detect *P. parva* DNA reliably from water samples was confirmed in aquarium trials (1 fish in 44-litre tanks) and in a field survey conducted in ponds where the species was known to occur (Davison et al. 2016).

123 **Study site and field sampling protocol**

124 The recreational angling venue, which was located in Kent, South-east England (Latitude
125 51°N, longitude 0°E), has no direct hydrological connections with an adjacent stream nor are
126 any of the seven angling ponds connected (Fig. 1). A single specimen of *Pseudorasbora*
127 *parva* was first captured in one of the angling ponds (area = 1.4 ha) in April 2004 but
128 reported in the angling press to be a young grass carp *Ctenopharyngodon idella* (fishery
129 owners, pers. comm.). An attempt to eradicate *P. parva* from this pond began in 2011 under
130 the guidance of an independent fisheries consultant (commissioned by the fishery owners).
131 From 2011 to July 2016, this consisted of intensive depletion using cylindrical fish traps (i.e.
132 60 by 30 cm crayfish traps with conical funnel entrance and fitted with 5 mm mesh netting).
133 The depletion trapping was complemented by repeated, high density (116 $kg \cdot ha^{-1}$) stocking of
134 a native predatory fish, Eurasian perch (*Perca fluviatilis*) – a biocontrol method that has been
135 demonstrated to exert a top-down effect on *P. parva* abundance (Davies and Britton 2015;
136 Verhelst et al. 2016). Initial reports received by the authors indicated that by 2014 *P. parva*
137 were no longer being captured, however trapping data recently acquired from the venue's
138 owners revealed persistence of a very small number of *P. parva*, with the lowest capture
139 densities occurring after predator releases (Fig. 2).

140 To ensure a statistically robust eDNA sampling protocol, an *a priori* power analysis was
141 performed (http://homepage.stat.uiowa.edu/~rlenth/Power/). This suggested that water should
142 be collected from 24 sampling locations (12 littoral and 12 pelagic). At each sampling
143 location, four sub-samples should be taken for analysis, and at least two PCR amplifications
144 should be performed for each sub-sample. According to this protocol (corresponding to a
145 doubly nested design), at a level of significance $\alpha = 0.05$, statistical power (β) would equal
146 0.806 for the sampling zones, 0.978 for the water samples, and 0.999 for the sub-samples.

147 Accordingly, post-eradication assessment using eDNA analysis consisted of three
148 sampling steps (Sept 2014; Nov 2014; Feb–Mar 2015). Firstly, 24 1-L water samples were
149 collected on 16 Sept 2014 in the infested pond, namely from 12 littoral zone locations spread
150 equidistantly (40 m apart) around the pond shore and 12 from pelagic zone locations spaced
151 around the water body (Fig. 1). Secondly, water sampling was undertaken during a return
152 visit on 12 Nov 2014 at the six littoral sampling points in the infested water body closest to
153 those where eDNA of *P. parva* was detected during step one. No *P. parva* DNA was detected
154 in any of the 12 pelagic (mid-water) samples, so these pelagic sample locations were not
155 considered further in the eDNA analysis. The water sampling on 12 Nov 2014 was
156 complemented by intensive sampling, using the fish traps described above. Traps were
157 deployed for five days in late December 2014, ten days in early February and six days in
158 early April 2015 (Fig. 2). Thirdly, water samples (1 L) were collected in 2015 on 17 Feb, 19
159 Feb and 5 March from 12 littoral zone locations in each of the other six ponds (areas of 0.5 to
160 2.4 ha), as well as at eight locations along the longitudinal course (1.5 km) of the small
161 stream that runs adjacent to the ponds. Pelagic samples were not collected from the other six
162 ponds, as this would have required movement of the boat between the water bodies, thus
163 increasing the risk of cross contamination.
164 In all cases, water samples were collected using a 183 cm sampling pole with a 500 mL
165 polypropylene sampling cup attached (Camlab Ltd, Cambridge, UK). The sampling cup was
166 moved in a standardised manner from the bank (littoral samples) or boat (pelagic samples) to
167 the greatest extent reached by the pole, ensuring no contact with the bottom sediment. At
168 each sampling location, 1 L of water obtained using the sampling cup was poured into a
169 sterilized plastic bottle. Samples were then placed in individual plastic bags and immediately
170 refrigerated (4°C) for transportation back to the laboratory. On each sampling day, two
171 identical ‘blank samples’ (new sterilized bottles of de-ionised water from the laboratory),

opened briefly in the field, were handled and transported in the same manner as the pond samples. Between samples, the sampling pole and cup were disinfected using Microsol 3+ (Anachem Ltd, Luton, UK) and washed with de-ionised water.

Laboratory protocol

Within 24 hours of collection, the water samples were filtered through a 0.4 µm pore size polycarbonate filter of diameter 47 mm (Isopore, EMD Millipore, Darmstadt, Germany) using a vacuum pump (EMD Millipore). From each sampling location, four sub-samples of 100 mL were filtered. Between filtration of samples from each location, the filtering equipment was sterilized using Microsol 3+, and washed with de-ionised water, and at regular intervals during filtration, de-ionised water was run through the filtration system, with these samples analysed to detect any potential cross-contamination. The filters were immediately frozen at –80°C. DNA extraction from the filters took place within three months from initial sampling using a PowerWater DNA Isolation Kit (MO BIO, Carlsbad, CA, USA).

Conventional PCR amplifications were performed in 20 µL reaction mixtures, containing 6 µL of DNA template, 0.5 µM of each primer, 10 µL (= 50 units) HotStar Taq Plus DNA polymerase (Qiagen Fast Cycling PCR Kit) and 2 µL CoralLoad Fast Cycling Dye (Qiagen). The cycling conditions employed were an initial denaturation step at 95°C for 5 min, followed by 32 cycles of denaturation (96°C; 5 s), annealing (61°C; 5 s) and extension (68°C; 12 s), with a final extension at 72°C for 1 min. Amplified PCR products were visualised using electrophoresis on 2% agarose gel, stained with SYBR Gold Nucleic Acid Gel Stain (Invitrogen, Paisley, UK). Three replicate PCRs were conducted for each 100 mL sub-sample, with each one including a negative control (de-ionised water) and a positive control (tissue-extracted *P. parva* DNA). To confirm the identity of sequences amplified from the

pond samples, PCR products from the positive sampling points were purified (Nucleospin Gel and PCR Cleanup) and sequenced by a commercial service (Eurofins Genomic Services Ltd, Wolverhampton, UK).

To confirm that negative results were not detection errors ('false negatives') caused by PCR inhibition, additional PCRs were conducted using the PCR protocol described previously (Jane et al. 2015; Adrian-Kalchhauser and Burkhardt-Holm 2016). PCRs were performed using an eDNA sample (6 µL) from a single location within each pond that was spiked with 2 µL of genomic *P. parva* DNA (0.01 ng/µL). The strength of the resultant electrophoresis gel band was compared visually with that from the same quantity of *P. parva* DNA amplified in deionised water alone (i.e. without sample). As these PCRs indicated the presence of inhibition, a further set of PCRs were undertaken in which the extracted samples (one sub-sample from each sampling location) were re-analysed following a 1:5 dilution in deionised water, a technique used to combat inhibition by diluting the inhibitory compounds (McKee et al. 2015). Three replicate PCRs were conducted on these diluted samples. To assess whether inhibition was still occurring following the 1:5 dilution, three replicate PCRs per pond were conducted in which a spike of 0.02 ng of tissue-extracted *P. parva* DNA was added.

Filtration, extraction, PCR preparation and post-PCR analysis were undertaken in separate rooms of a laboratory dedicated to molecular biology, observing strict anti-contamination procedures (no transfer of equipment between rooms; changing of labcoats when moving between rooms; thorough cleaning of all equipment and surfaces before and after use).

Results

In the initial sampling step, of the infested water body only, *P. parva* DNA was detected at three of the 12 littoral zone locations (Table 1). These sampling locations came from adjacent

222 locations at one end of the pond (Figure 1). DNA of *P. parva* was not detected in any of the
223 100 mL sample replicates collected from the pelagic zone. Spiking tests indicated a small
224 level of inhibition occurring in pelagic and littoral samples. Two samples contained the
225 minimum quantity of DNA required for sequencing, which confirmed the identity of the
226 eDNA as that of *P. parva*. Both sequences showed a 100 % match with 34 sequences of *P.*
227 *parva* registered in the Genbank database (e.g. accession number HQ960448).
228 In the second sampling step, repeat sampling and eDNA analysis of water from the
229 locations where *P. parva* eDNA had been detected in step one provided further confirmation
230 of the species' presence. This corroborated the trapping data recently acquired from the
231 venue's owners (Fig. 2).
232 In the third sampling step, all sample replicates from the other six angling ponds and from
233 the adjacent small stream proved negative for *P. parva* eDNA. Spiking tests indicated a small
234 level of inhibition occurring in all six ponds. Following the 1:5 dilution of extracted samples
235 to combat the detected inhibition, no further inhibition was detected. All samples that had
236 previously shown negative for *P. parva* DNA (i.e. previously negative littoral locations and
237 pelagic locations from the infested pond, and all samples from the *a priori* non-infested
238 ponds) also proved to be negative following the 1:5 dilution. These results suggest that the
239 level of inhibition occurring in the samples was not sufficient to mask the presence of DNA
240 during the first analysis.
241
242 **Discussion**
243 The current study demonstrates that eDNA surveys are a valuable method for post-evaluation
244 of eradication attempts, with equal, if not greater, power to detect remnant populations of
245 target species than conventional survey methods. Water samples subjected to eDNA analysis
246 confirmed the persistence of a small population of *P. parva* in the infested pond, as indicated

247 from trapping results (Britton et al. 2011b). In the other water bodies, eDNA analysis
248 corroborated trapping results for the other six angling ponds and electrofishing results for the
249 adjacent stream, that indicate it is unlikely the species was present at the time of sampling.
250 Small-bodied fishes at low population densities can often be difficult to detect, and
251 imperfect detection using conventional methods (i.e. electric fishing and trapping) has
252 previously been demonstrated for *P. parva* in 100 m^2 ponds (Britton et al. 2011b). At low
253 population abundances, eDNA surveys may represent the most effective method of
254 confirming the presence of a fish species. For example, eDNA sampling detected the
255 presence of European weather loach (*Misgurnus fossilis*) in a location where it had not been
256 recorded for 13 years using traditional methods, including fish traps, electrofishing and seine
257 nets (Sigsgaard et al. 2015). In the present study, the spatial heterogeneity of the positive
258 eDNA detections is likely to reflect the heterogeneous distribution of the target species,
259 which has been recorded previously (Li et al. 2010; Davison et al. 2016). The lack of
260 detections from the open water sampling locations is indicative of a distribution favouring
261 shallow vegetated areas in the littoral zone (as previously shown for *P. parva*: Li et al. 2010),
262 or an alternative favoured habitat type that is present in only a few isolated locations around
263 the pond. The trapping of 78 specimens in the vicinity of these sampling points (seven
264 months after the initial water sample collection), suggests that a small, localised population in
265 this area was the most likely source of the detected eDNA.
266 Spatial heterogeneity of eDNA is common in lentic water bodies (e.g. Eichmiller et al.
267 2014), emphasising the need for sufficient water samples to be collected (with adequate
268 spatial coverage) to increase the likelihood of detection of localised species in low
269 abundance. In the present study, only five positive detections resulted from 96 sub-samples of
270 water from 24 locations in the infested lake. Detection power could potentially have been
271 improved by modifying the PCR protocol, such as increasing the number of cycles

(Rameckers et al. 1997). The sensitivity of detection could arguably be increased by using quantitative real-time PCR (qPCR) protocols, for which higher levels of sensitivity have been reported (Tréguier et al. 2014; Biggs et al. 2015). However, in mesocosm trials, no difference between qPCR and conventional PCR was found in the detection of DNA of target species present at low density (Nathan et al. 2014). A practical consideration is that conventional PCR is financially less costly than qPCR, and therefore more likely to be available to those tasked with the management of invasive species (Davison et al. 2016).

The lack of detection of *P. parva* DNA in the six other lakes on site serves to corroborate the species' absence in angler's catches and conventional surveys undertaken before and after the eDNA survey (fishery owners, pers. comm.). Indeed, no *P. parva* were observed or captured in the adjacent stream during an electrofishing survey carried out a few months after the water samples for eDNA analysis were collected (Environment Agency, pers. comm.). Whilst caution is always needed when declaring a species to be absent on the basis of absence of detection, regardless of the survey method used (Mackenzie 2005; Kéry & Schmidt 2008), the statistically-rigorous sampling protocol used here suggests that it is unlikely that *P. parva* is present in the other nearby, but unconnected, ponds and the stream. PCR-inhibiting compounds in the water are a potential cause of false negatives, but in this case study the detected inhibition was not sufficient to affect the results. It does demonstrate, however, the importance of incorporating steps in laboratory protocols to assess the extent of inhibition, and if necessary to overcome inhibition by methods such as dilution of samples or addition of bovine serine albumin (Deiner et al. 2015; McKee et al. 2015).

The risk of false positives also needs to be considered when basing management decisions on the results of eDNA surveys. Positive detections should not necessarily be taken as an indication of presence of live organisms, as DNA could enter the water from other sources, e.g. decaying corpses or bird faeces (Merkes et al. 2014; Dunker et al. 2016). Before costly

297 management action is taken, ‘ground truthing’ (i.e. capture of live individuals) is
298 recommended to corroborate eDNA detection, such as was the case in the present study.
299 The present study demonstrates the applicability of eDNA surveys to assess the efficacy
300 of eradication attempts in aquatic environments, providing additional support for studies
301 elsewhere in which eDNA analysis was reported to be more sensitive than conventional
302 methods for detecting species present in low abundance. Accurate assessments of the success
303 of eradications is important; the continuation of a monitoring programme after the final
304 individuals have been removed can be costly, whilst conversely the premature declaration of
305 success and resultant cessation of monitoring can be even more costly and potentially nullify
306 previous efforts (Rout *et al*. 2009, 2014). Surveys based on eDNA analysis are therefore an
307 important tool to assist the decision-making process as regards the management of non-native
308 species, both for early detection and rapid response, as well as for the assessment of
309 eradication success. To this end, a nested quantitative PCR protocol is currently being tested
310 in still and running waters for such applications to enhance the sensitivity of the analysis.
311

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319

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Figure legends

Fig. 1. Schematic map (Scale bars = 100 m) of the study site in the English county of Kent, showing location of the seven ponds and adjacent stream. In the infested lake (inset), pelagic sampling locations are indicated with small, open circles, whereas littoral sampling locations (open squares) are numbered (see Table 1), the filled squares indicating locations where positive detections of *P. parva* DNA occurred in the initial sampling survey (September 2014). Locations 1 and 10 also came up positive in November 2014 (Table 1).

Fig. 2. Numbers of topmouth gudgeon *Pseudorasbora parva*, calculated on a per trap per month basis, captured by fishery staff between 2011 and 2016 using fish traps (see Methods) placed around the water body's banks each sampling excursion. The arrows indicate dates of predator biocontrol release, i.e. 400, 200, 400, and 246 Eurasian perch *Perca fluviatilis* (left to right, respectively) of 6–9 cm total length.