A simple, rapid method for detecting seven common invasive fish species in Europe from environmental DNA

Clusa L*, García-Vázquez E

Department of Functional Biology, University of Oviedo. Avda. Julian Claveria s/n 33006-Oviedo, Spain

ABSTRACT

1. Biological invasions are a global threat to biodiversity, and many come from deliberate introductions.
2. The American freshwater fish *Micropterus salmoides* and *Ameiurus* sp (*A. melas* and *A. nebulosus*) were introduced to Europe for recreational fishing; *Gambusia holbrooki* and *G. affinis* for mosquito population control and *Lepomis gibbosus* as an ornamental species. The Asiatic *Pseudorasbora parva* was acquired inadvertently as an accompanying species of fish consignments.
3. This paper presents a novel approach for detecting these species directly from water samples based on a panel of five taxon-specific primers within the 16S rDNA.
4. The primers were validated from tissue, in aquarium experiments, and from Ebro River water samples (Spain). With a simple PCR protocol followed by visualization in agarose gel or capillary electrophoresis it was possible to detect these species from environmental DNA concentrations as low as 0.89 to 100pg mL^{-1}.
5. This sensitive and economical tool can be useful to control the European invasions of these species and preserve the native biodiversity.

*Corresponding author: Laura Clusa, lauraclusa@gmail.com, orcid.org/0000-0002-3503-2331.

Keywords: river, biological control, fish, alien species, species-specific primers; eDNA; PCR; 16S rDNA.

Introduction

Biological invasions are an important threat to biodiversity (Chown et al., 2015), since they often result in local extinctions or extirpation of autochthonous species (Clavero & García-Berthou, 2005). Aquatic species are translocated worldwide for various purposes, from fishing to aesthetic pleasure (Havel, Kovalenko, Thomaz, Amalfitano, & Kats, 2015). In Europe, freshwater fish are the most frequently introduced aquatic species (García-Berthou et al., 2005). The north American largemouth black bass *Micropterus salmoides* and bullhead catfish *Ameiurus* spp. (*A. melas* and *A. nebulosus*) were introduced into many European waters for recreational fishing (Copp et al., 2016; Savini et al., 2010). The mosquitofish *Gambusia holbrooki* and *G. affinis* were widely introduced to Europe for mosquito control (Pyke, 2008), from their native range in North America (Sanz et al., 2013). Another cause of exotic fish spread is imports for aquarium trade that are often released into the wild. An example is the introduction of north American *Lepomis gibbosus* (pumpkinseed) to Europe as an ornamental species (Maceda-Veiga, Escribano-Alacid, de Sostoa, & García-Berthou, 2013). An unexpected impact of non-native fish farming in Europe was the inadvertent introduction of highly invasive accompanying species as contaminants in farm fish consignments. For example the topmouth gudgeon (or stone moroko) *Pseudorasbora parva* was transported together with Chinese carp from Asia to Romania in 1960, and today it is present in almost every country in Europe (Gozlan et al., 2010; Simon, Gozlan, Britton, van Oosterhout, & Hänfling, 2014). The EU regulation No 1143/2014 of 22 October 2014 on Invasive Alien Species (http://ec.europa.eu/environment/nature/invasivealien/index_en.htm) states in its Article 14 that Member States should establish a surveillance system to detect rapidly the appearance of any invasive alien species in the environment of a Member State. Rapid detection is indeed important because biological invasions are better controlled in the initial invasion stages (e.g. Blackburn et al., 2011).

Ecological impacts of the above mentioned species have been demonstrated in Europe, where they compete with native species for habitat and food resources (Ribeiro & Leunda, 2012). *Lepomis gibbosus* exhibits aggressive behavior when competing for food and territory (Almeida, Merino-Aguirre, Vilizzi, & Copp, 2014). *Gambusia* species affect native fauna such as invertebrates and amphibians through predation (Pyke, 2008; Remon, Bower, Gaston, Clulow, & Mahony, 2016). They alter the plankton communities and subsequently the whole ecosystem (Hurlbert & Mulla, 1981; Hurlbert, Zedler, & Fairbanks, 1972). Introduced black bass (*M. salmoides*), an aggressive predator, usually affects populations of small native fishes by predation, sometimes causing their decline or extinction (Maezono & Miyashita, 2002; Weyl & Lewis, 2016). *Ameiurus* sp may also have adverse ecosystem effects by increasing turbidity (Braig & Johnson, 2003). Moreover, these invasive species are hosts of many parasites. For example, *L. gibbosus* introduced new parasites (*Onchocleidus* sp) in Norway (Sterud & Jørgensen, 2006). *Pseudorasbora parva* carries many parasites, such as the rosette agent (*Sphaerothecum destruens)* (Gozlan et al., 2010; Pinder, Gozlan, & Britton, 2005), being capable of transmission to native fish species (Gozlan, St-Hilaire, Feist, Martin, & Kent, 2005).

Eradication of these invasive species, when possible, may allow the recovery of native fauna. This happened in 11 small ponds from Oshu city, north-eastern Japan, after eradication of *M. salmoides* (Tsunoda, Mitsuo, Ohira, Doi, & Senga, 2010). In one lake from the Lake District in the north-west of UK, populations of native *Rutilus rutilus* and *Abramis brama* increased after *P. parva* eradication (Britton, Davies, & Brazier, 2009). Early detection of non-native fish is crucial for a rapid and efficient response to prevent further establishment or spread.

In the last few years, environmental DNA (eDNA) survey methods have proved a promising tool for detecting and surveying invasive species in aquatic ecosystems. Metazoans can be detected from their DNA released into the environment through skin flaking and sloughed cells, mucus excretion and defecation (Goldberg, Strickler, & Pilliod, 2014). This method seems to be sensitive and efficient, and unlike most classic sampling methods (electrofishing, netting) it does not disturb the aquatic fauna (Blanchet, 2012; Ficetola, Miaud, Pompanon, & Taberlet, 2008; Thomsen et al., 2012). Specific PCR primers used on eDNA have been successful in detecting a number of species from water samples. Examples are molluscs (Ardura et al., 2015; Devloo-Delva et al., 2016; Clusa et al., 2016), fishes (Furlan & Gleeson, 2016; Gustavson et al., 2015; Takahara, Minamoto, & Doi, 2013; Adrian-Kalchhauser & Burkhardt-Holm, 2016; Uchii, Doi & Minamoto, 2016), amphibians (Ficetola et al., 2008; Pilliod, Goldberg, Arkle, & Waits, 2014), reptiles (Piaggio et al., 2014; Davy, Kidd, & Wilson, 2015) and mammals (Foote et al., 2012; Ushio et al., 2017).

The aim of this study was to check the potential of a simplified PCR-based method for early alert of seven common invasive fish species *A. melas*, *A. nebulosus*, *G. affinis*, *G. holbrooki*, *M. salmoides*, *L. gibbosus* and *P. parva* from water samples. If successful, the method could be applied by managers for river surveillance. For this purpose, new specific primers were developed and tested experimentally in vitro and in aquarium, as well as from field water samples. The seven species have been reported from many European countries, including Spain (Elvira & Almodóvar, 2001; Leppäkoski, Gollasch, & Olenin, 2002). They are in the Spanish official list of invasive alien species (Spanish Royal Decree 630/2013 of 2 August 2013, https://www.boe.es/buscar/doc.php?id=BOE-A-2013-8565). They all occur in Ebro River (north-east Spain), as reported in the webpage of the Regional Government of Aragón; thus, the new tool was field tested from Ebro River waters. The method could be also applied in other European waters for surveillance of these invasive alien species.

Materials and Methods

Species studied

For confirming the adequacy of the species choice an exhaustive search was performed in three databases for invasive species: EASIN (European Alien Species Information Network; http://easin.jrc.ec.europa.eu/ accessed in November 2016), DAISIE (Delivering Alien Invasive Species Inventories Europe; http://www.europe-aliens.org/ accessed in November 2016) and the GISD (Global Invasive Species Database; http://www.iucngisd.org/gisd/ accessed in November 2016) of the IUCN (International Union for Conservation of Nature). The criterion of choice used was species invasiveness to European countries. The species selected were those invasive to a higher number of European countries (the top five). The non-native species invasive to Europe were compiled (Table S1) and the five taxa of choice in this study (*Ameiurus* sp, *Gambusia* sp, *L. gibbosus*, *M. salmoides* and *P. parva*) are listed there amongst the commonest invasive non-salmonid fish to European countries. In Spain there are 61 species from 24 fish families officially listed as native to the Iberian Peninsula, and 36 non-native species from 15 families including the aforementioned ones (Table S2).

Design of species-specific primers

The method applied is based on conventional PCR. The 16S rRNA gene was chosen for the design of the primers based on reference nucleotide sequences from GenBank

(www.ncbi.nlm.nih.gov/) plus the sequences obtained in this study. 16S rRNA is a mitochondrial gene, present in higher copy number than nuclear genes in eDNA samples (Thomsen, & Willerslev, 2015), it does not show great variation within species but it shows high variation between closely species, especially in fishes (Maretto, Reffo, Dalvit, Barcaccia, & Mantovani, 2007; Vences et al., 2016) and the number of sequences for fishes in databases is similar to other mitochondrial genes as COI or cytochrome b (Machida, Leray, Ho, & Knowlton, 2017). Sequences of this gene (either individual 16S DNA sequences or complete mitochondrial genomes) for the target fish and other species of a wide range of aquatic taxa were downloaded from GenBank and aligned with the ClustalW application included in BioEdit (Thompson, Higgins, & Gibson, 1994). Polymorphisms were analyzed with DNASP software (Rozas, Sánchez-DelBarrio, Messeguer, & Rozas, 2003). The different haplotypes were visualized employing BioEdit Sequence Alignment Editor (Hall, 1999). Within the 600 nucleotide amplicon obtained with the universal primers designed by Palumbi et al., (2002), regions conserved within each of the target species (identical in all sequences of that species) but different in the rest of reference species collected were located. These regions were used to design the set of specific primers (Table 1).

Table 1: Taxon-specific primers designed in this study. Primer's sequence, annealing temperature, Mg^2 concentration, expected amplicon size (in base pairs); initial DNA concentration employed for testing sensitivity of the primer pairs (stock), maximum dilution (last dilution) and corresponding DNA concentration (detection limit) for which is possible to obtain a PCR product visible in agarose gel with the primer pairs in the conditions assayed.

Species	Primer	Sequence (5'-3')	Annealing Temperature	$[Mg^{2+}]$	Amplicon size	Stock	Last dilution	Detection limit
Gambusia sp	Ga-16S-F	GRAACCAACTGACCCCTGCTT	68°C	1mM	117pb	0.535 µg mL^{-1}	1/600 000	0.89 pg mL^{-1}
	Ga-16S-R	GTTTTGTGAGCTGCGGCTCTWTA						
Micropterus salmoides	MiSa-16S-F	WCATCCCRAAACAAAGGGCY	68°C	2mM	142pb	0.57 µg mL^{-1}	1/100 000	5.7 pg mL^{-1}
	MiSa-16S-R	AATTCTGTTCATTAGAGCGGAGG						
Ameiurus sp	Am-16S-F	CGTCAAGAACYCAGTTRAACT	65°C	1mM	134pb	0.7 µg mL^{-1}	1/5 000	140 pg mL^{-1}
	Am-16S-R	GWTTCTGYGACTTAGAGTTGTCA						
Pseudorasbora parva	PsPa-16S-F	GTTTAAYCATGTTAAACAACTTAT	58°C	2.5mM	192pb	0.5 µg mL^{-1}	1/5 000	100 pg mL^{-1}
	PsPa-16S-R	TTCGTTGATCGACTATGTGT						
Lepomis gibbosus	LeGi-16S-F	GGACACGGGGCTAAACCAAAT	68°C	1mM	113pb	0.535 µg mL^{-1}	1/600 000	0.89 pg mL^{-1}
	LeGi-16S-R	GGGCTCTTAGTTGTGGAATTGCA						

Fish tissue and water sampling in field and aquarium

Tissue samples were provided by Centro de Acuicultura Vegas del Guadiana. Genetic barcoding was done using cytochrome c oxidase subunit I (COI) (Geller, Meyer, Parker, & Hawk, 2013) and 16S rRNA genes (Palumbi et al., 2002) in order to confirm the species of each tissue sample.

To sample eDNA, 1L of water was collected in sterile plastic bottles from each sampling point, in both aquariums and river. Water samples were vacuum filtered using

the Supor®-200 Membrane Filter (Pall Corporation) with 0.2 µm pore size (Turner et al., 2014) and a filter holder. The filtration apparatus was cleaned with 10% bleach, rinsed with distilled water and sterilized under UV light for 20 minutes between filtrations. Filters were put individually within 15mL tubes and stored at -20°C until DNA extraction.

DNA extraction and PCR conditions

DNA from tissue samples was extracted using Chelex resine as described by Estoup, Largiader, Perrot, & Chourrout, (1996). DNA from water samples was extracted directly from the filters with the PowerWater® DNA Isolation Kit (Mobio laboratories) following manufacturer's recommendations. The eDNA extractions were done under sterile conditions, in a laboratory unit where there were no other tissue samples, inside a PCR laminar flow cabinet treated with ultraviolet light to avoid any contamination of the environmental DNA. As a negative control for extraction (blank sample) 1L of distilled water was treated equally as the samples for all the processes and included in each analytical step to be sure that contamination did not occur, as described in Clusa, Ardura, Fernández, Roca & García-Vázquez (2017).

For positive control samples, DNA extracted from tissue of *A. melas, G. holbrooki, L. gibbosus, M. salmoides* and *P. parva* was PCR-amplified with the newly developed primers (Table 1). For confirming that cross-amplification negative results were not due to PCR failure, universal primers for the 16S rRNA gene (Palumbi et al., 2002) were employed for PCR amplification on the same samples.

The amplification reaction with the taxon-specific primers from tissue DNA was performed in a total volume of 20µL, including Green GoTaq® Buffer 1X, $MgCl_2$, 0.25mM dNTPS, 1µM of each primer, 2µL of template DNA and 0.65 U of DNA Taq polymerase (Promega). The PCR conditions were the following: an initial denaturation step at 95ºC for 5min, 35 cycles at 94ºC for 30s, annealing at the temperature of choice for 30s and elongation at 72ºC for 30s. A final step of elongation was set at 72ºC for 10min. Different annealing temperatures and $MgCl_2$ concentrations for each pair of primers were assayed (Table 1). PCR products were visualized in 2% agarose gels with 2.5µL of SimplySafe™.

In the case of DNA extracted from water samples, the PCR conditions were the same as described above with some minor modifications. Fifty cycles were used instead of 35 and 6µL of DNA template. BSA (200ng mL^{-1}) was added in the PCR mix to avoid the effects of inhibitors in the sample (Jiang, Alderisio, Singh, & Xiao, 2005). In addition to the blank sample, negative controls containing only PCR reagents and distilled water were included in every PCR.

In silico and *in vitro* validation of designed primers

The new taxon-specific primers were tested first *in silico* with the BLAST tool in the NCBI webpage (Altschul, Gish, Miller, Myers, & Lipman, 1990) to confirm they aligned significantly only with the target species. To validate the marker *in vitro*, cross-amplification tests were performed using tissue DNA of different fish species occurring in Spanish waters. False positives may occur from native species of the same genus, perhaps of the same family. Three of the four families containing the species considered in this study (Centrarchidae, Ictaluridae and Poeciliidae) are non-native to Europe (Freyhof & Brooks, 2011), thus native species of such families do not occur from Spanish waters and false positives are not expected. However, there are Iberian native species from the Cyprinidae family, although not from the same genus considered in this study (*Pseudorasbora*), and other exotic Cyprinidae genera as well (Table S2). Thus for cross-

amplification two native cyprinids (*Phoxinus phoxinus*, *Squalius pyrenaicus*) and two non-native cyprinids (*Carassius auratus*, *Leuciscus idus*), and native species representative of three families common in Spanish waters: *Salmo trutta* (Salmonidae), *Platichthys flesus* (Pleuronectidae) and *Dicentrarchus labrax* (Moronidae) were tested.

The primers developed were tested for cross-amplification with the seven species above and the five target species of this study (*Ameiurus melas*, *Gambusia holbrooki*, *Lepomis gibosus*, *Micropterus salmoides*, *Pseudorasbora parva*).

The detection limit of PCR with taxon-specific primers, visualized in agarose gels, was determined from serial dilutions of a known DNA concentration for each species. The concentration previous to that where no amplification was observed in agarose gel was considered the detection limit. DNA concentration was measured with a fluorometer Qubit® dsDNA BR Assay.

In situ validation of designed primers

The method was also validated in environmental DNA from controlled aquarium water samples provided by Zaragoza's city Aquarium and Ebro's Delta Ecomuseum. Water samples (1L) from tanks containing individuals of each of the studied taxa and other fish were analyzed with the five newly designed primers (Table 2). The five sets of primers were used for each tank.

For validation with field environmental samples the method was applied in Ebro River as a case study. The seven species have been reported at several places in the Ebro basin (Ministerio de Medio Ambiente 2007). At 930 kilometers it is the second longest river in the Iberian Peninsula, and the second as well by flow rate, with an average discharge of 400 m^3 s^{-1} (Confederación Hidrográfica del Ebro, 2016).

Table 2: Aquarium experiments. Water volume in the aquarium (in L), number and size of individuals of the target species, other species present in each aquarium together with the target one (number of individuals in parenthesis).

Experiment	Volume (L)	Target species	Number of individuals (length in cm)	Other species (Number of individuals)
Aquarium 1	800	*Gambusia holbrooki*	10 adults (≤3cm)	*Salaria fluviatilis* (35)
Aquarium 2	6500	*Micropterus salmoides*	2 adults (45cm)	*Anguilla anguilla* (27), *Emys orbicularis* (3)
Aquarium 3	7640	*Ameiurus melas*	2 adults (23-25cm)	*Barbus graellsii (17)*, *Cyprinus carpio* (1)
Aquarium 4	2460	*Lepomis gibbosus*	4 adults (5-7cm)	*Barbus graessi* (8), *Gobio lozanoi* (10), *Parachondrostoma arcasii* (2)
Aquarium 5	200	*Pseudorasbora parva*	15 adults (8-12cm) and 7 juveniles (<8cm)	-
		Gambusia holbrooki	1 adult (2cm)	-

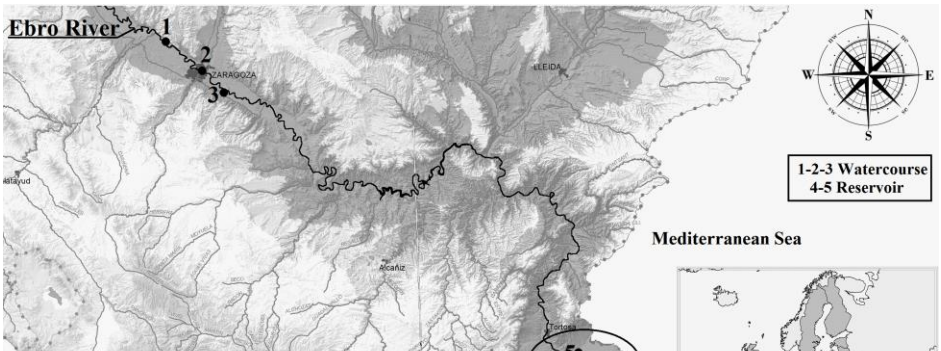

Figure 1. Map of Ebro River and its location in Iberian Peninsula. The Ebro River is highlighted in black and the five sampling points are shown (downloaded from Confederación Hidrográfica del Ebro).

In December 2015, water samples were taken from five points along Ebro River. Three of them were sampled from running waters, far away from reservoirs, and two inside the river delta (Figure 1). The three samples from running waters were taken near the largest city crossed by the river (Zaragoza): one upstream from the city in Utebo (sampling point #1), another in the middle of Zaragoza city (sampling point #2) and the third one in Movera downstream (sampling point #3). The two samples collected in the Ebro River delta were point #4 and point #5 in two ponds, where Caiola and De Sostoa (2002) reported the occurrence of *P. parva*.

Two replicates of 1L water were collected with sterile bottles from each sampling point, putting the bottle as close to the bottom substrate as possible. They were immediately transported to the laboratory on ice and then frozen. At point #1, a survey was carried out along the riverside using a landing net. In total, 100 m were surveyed from the riverside. At the rest of the locations manual netting was not possible owing to very high river flow and rapid currents.

The primers were assayed twice to confirm the results: two replicate PCRs were done on each eDNA sample. All the positive bands found were purified, sequenced and the species confirmed by BLAST against GenBank.

Phylogenetic analysis of the DNA fragment amplified from genus-specific primers

In the case of the two genus-specific primers (one for the genus *Gambusia* and the other for the genus *Ameiurus*) additional phylogenetic analysis was done in order to check if the primers distinguish between the different species of a genus. Different reference sequences of *G. holbrooki, G. affinis, A. melas, A. nebulosus, A. bruneus, A. natalis* and *A. catus* were downloaded from GenBank. The sequences obtained in this study as well as the additional reference sequences were aligned with the ClustalW application included in BioEdit (Thompson et al., 1994). A Neighbour-Joining tree (Saitou & Nei, 1987) was built using MEGA 6.0 (Tamura, Stecher, Peterson, Filipski, & Kumar, 2013), with 10000 bootstraps. The evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura, Nei, & Kumar, 2004)

Results

Design of specific primers and experimental validation

Taxon-specific primers were designed for the analyzed taxa (Table 1). For *Gambusia* sp and *Ameiurus* sp the primers were genus-specific and amplified from the two species of each genus listed as invasive to Europe: *G. holbrooki* and *G. affinis*, *A. melas* and *A. nebulosus*.

From *in silico* BLAST assays, the new primers retrieved significant alignments only fwith the species for which they were designed. Consistently with these results, cross amplification was not found for the assayed species and for each pair of primers positive PCR amplification occurred only from DNA of the target species (Figure 2). A single clear band of the expected size was obtained with the primers designed for each target species, and the sequence obtained from the bands corresponded to the targeted species and gene (Table S3). Positive amplification of 16S rRNA gene with universal primers (Palumbi et al., 2002) was found for all the samples used in cross amplification tests (Figure 2A), confirming that DNA was of sufficient quality for successful PCR analysis. Sequences from genetic barcoding of COI and 16SrRNA genes of each tissue sample are available in GenBank (accession numbers KU510486, KU510498, KU510509 and from KY231824 to KY231835).

The threshold of detection for PCR product visualization in agarose gels ranged from 100pg mL^{-1} for *P. parva* to 0.89pg mL^{-1} for *Gambusia* sp and *L. gibbosus* (Table 1).

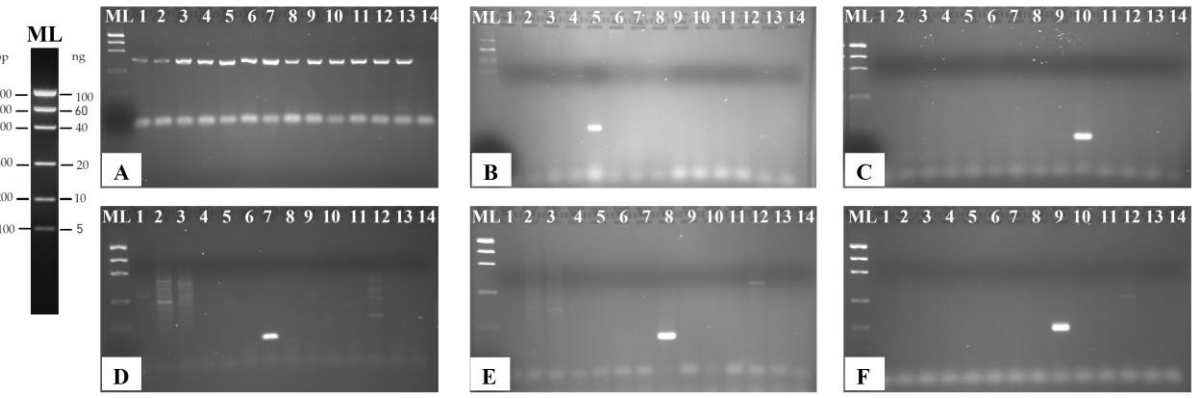

Figure 2. Agarose gels (2%) showing the results of cross-amplification experiments for each specific marker. 16S rDNA PCR amplified with: A) universal primers (Palumbi et al., 2002); specific primers for *Ameiurus* sp. (B); *Gambusia* sp. (C); *Lepomis gibbosus* (D); *Micropterus salmoides* (E); *Pseudorasbora parva* (F). Lanes (from 1 to 14) in all gels are: Ladder (ML), 1-*Salmo trutta*, 2-*Dicentrarchus labrax*, 3-*Platichthys flesus*, 4-*Alburnus alburnus*, 5-*Ameiurus melas*, 6-*Carassius auratus*, 7-*Lepomis gibbosus*, 8-*Micropterus salmoides*, 9-*Pseudorasvora parva*, 10-*Gambusia holbrooki*, 11-*Phoxinus phoxinus*, 12- *Leuciscus idus*, 13-*Squalius pyrenaicus*, 14- Negative control.

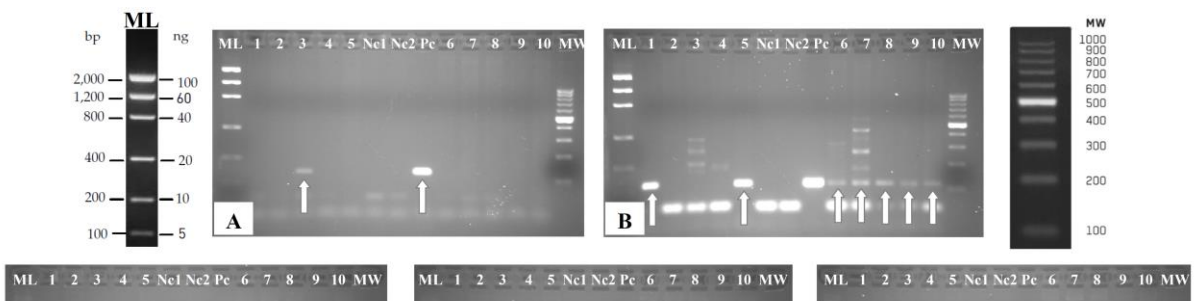

Figure 3. Agarose gels (2%) showing PCR products from eDNA for each specific marker. A) *Ameiurus* sp; B) *Gambusia* sp; C) *Lepomis gibbosus*; D) *Micropterus salmoides*; E) *Pseudorasbora parva*. Lanes (from 1 to 15) in all gels are: Ladder (ML), aquarium 1 (1), aquarium 2 (2), aquarium 3 (3), aquarium 4 (4), aquarium 5 (5), Ebro River point 1 (6), Ebro River point 2 (7), Ebro River point 3 (8), Ebro River point 4 (9), Ebro River point 5 (10). Negative controls are indicated as (Nc1 and Nc2), negative control for extraction and negative control for PCR, respectively. Positive control with tissue DNA of each species (Pc).

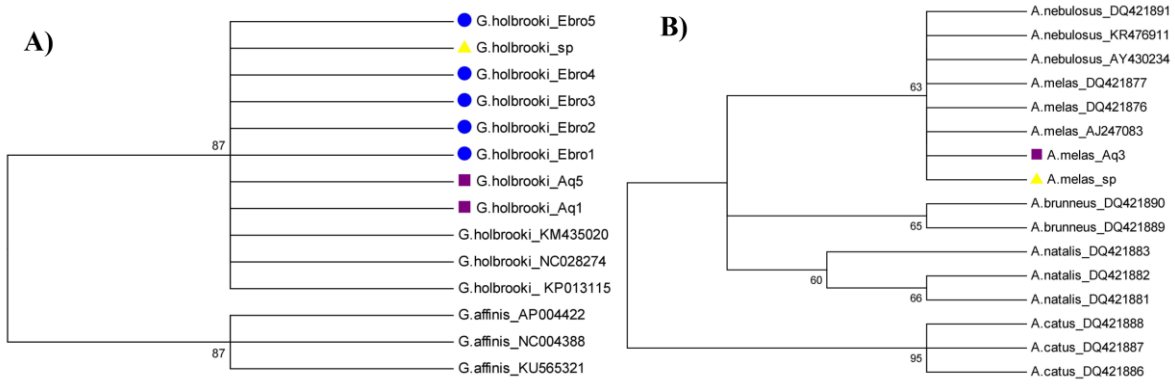

Figure 4. Phylogenetic trees reconstructed from sequences obtained in this work and references from GenBank (the accession number is indicated). Tissue positive samples are indicated with a yellow triangle, aquarium samples are indicated with a purple square and Ebro River samples are indicated with a blue circle. A) *Gambusia* sp (76 nucleotides), B) *Ameiurus* sp (94 nucleotides).

For the experimental validation in the aquaria, each species was detected only in water from the tank where it was present. PCR from specific primers was successful even in the case of the tank containing only one small individual of *G. holbrooki* in Aquarium number 5 (Figure 3). All the bands marked with an arrow in Figure 3 were sequenced, and the species was confirmed by BLAST (Table S3 and DDBJ accession numbers LC198795- LC198812). The negative controls for extraction (Nc1 in Figure 3) were clean and contamination along the process could be discarded.

The phylogenetic analysis showed that the sequences obtained with the two genus-specific primer pairs could separate the two *Gambusia* species, but could not distinguish the two invasive species targeted within the genus *Ameiurus*. However, these two species (*A. melas* and *A. nebulosus*) clustered separately from the rest of *Ameiurus* species (*A. brunneus, A. natalis* and *A. catus*), that are exotic to Europe but not considered invasive (Figure 4A for *Gambusia* and 4B for *Ameiurus*).

Assays in field water samples: Ebro River

The results of Ebro River (Table 3, Figure 3) revealed DNA of three of the target taxa from the water samples analyzed: *Gambusia* sp, *L. gibbosus* and *P. parva*. Positive detection was obtained in the two replicates of eDNA samples taken. *Gambusia* sp were found from all the sampling points. *Lepomis gibbosus* was found from sampling point #1, and *P. parva* from point #5, in Ebro River delta where it had been reported by Caiola & De Sostoa (2002). For *Ameiurus* sp and *M. salmoides*, positive PCR amplification was not found from any Ebro River sample.

The positive bands were sequenced and are available in Table S3. The sequences amplified with *Gambusia*-specific primers from river water samples corresponded to the species *G. holbrooki*. The sequences clustered together with *G. holbrooki* reference sequences KM435020, NC028274, KP013115, supported by a robust bootstrap of 87 (Figure 4A).

Six *G. holbrooki* individuals were caught manually from sampling point #1, the only point where land nets could be used. Their physical occurrence confirmed the validity of eDNA analysis for detecting this species from running waters.

Table 3: Ebro River field eDNA results. Sampling points along the Ebro River and their coordinates, and PCR amplification results obtained with the taxon-specific primers designed in this study. Positive PCR amplification is marked with X. Negative PCR is indicated as "-".

Sampling points	Coordinates	*Ameiurus sp*	*Gambusia sp*	*Lepomis gibbosus*	*Micropterus salmoides*	*Pseudorasbora parva*
#1	41.736952N, -0.992233W	-	X	X	-	-
#2	41.658574N, -0.878066W	-	X	-	-	-
#3	41.632217N, -0.837865W	-	X	-	-	-
#4	40.64336N, 0.7104704E	-	X	-	-	-
#5	40.72397N, 0.721833E	-	X	-	-	X

Discussion

The set of specific primers designed and validated in this study has proved very sensitive for detection of seven of the commonest invasive species in Europe directly from water samples, and can be used for direct species detection from field water samples. Other specific primers for *P. parva* and *L. gibbosus* have been assayed experimentally in aquarium tanks (no running water), and in artificial ponds with known fish populations (Davison et al., 2016). In addition, other specific primers for *P. parva* designed by Keskin (2014) in the COI gene were successfully applied on river water samples. This study contains several innovations. This is the first case of primers validated for detecting *L. gibbosus* from running water samples; the first primers designed within the 16SrDNA gene for *P. parva*; and the first eDNA method at all, to our knowledge, for the other five species (*Gambusia* sp, *Ameiurus* sp and *M. salmoides*).

Finding positive amplification results from Ebro River running water was encouraging because it confirms the power of eDNA-based methodology. Despite high flow and rapid current in this river, it was possible to detect three different species directly from small volumes of running water. Turner et al. (2014) demonstrated that smaller pore

filters (0.2µm) can recover eDNA quantities from small water volumes (similar to the ones used in this study) equivalent to those obtained from filtration of larger water volumes through larger pore filters. Other studies have employed from 250 mL up to 5 L of water samples (Goldberg et al., 2016). The *Gambusia* primers enabled detection of a *G. holbrooki* population in a zone (points #1-3) where the river is wide (133 ± 24m), and where classic sampling is very difficult. The occurrence of *P. parva* in the river delta, earlier reported by Caiola and De Sostoa (2002), was also confirmed using eDNA and revealed that the population is still there 15 years later.

Regarding the sensitivity of the five sets of primers, the detection limit was in the range of pg mL^{-1}, similar to that described by Davison et al. (2016) for *L. gibbosus* and *P. parva* specific primers. Therefore, the method would be useful for detecting these species in early invasion stages, when the population size is still low and might be overlooked from traditional sampling methods. Owing to its sensitivity, the method could be applied to detect the seven invasive species in other European streams where they are suspected. It could be especially useful in large streams, such as Rhine River, which is connected to nearly all the large rivers in south-western, southern, central and eastern Europe and could be the entrance of these invasive species (Leuven et al., 2009). For Centrarchidae, Ictaluridae and Poeciliidae, which are non-native families of European rivers (Freyhof & Brooks, 2011), any positive result would indicate the occurrence of an exotic species. Sequencing the amplicon would confirm the identification of the non-native species and differentiate between congeneric species, except between the two *Ameiurus* species tested here.

The new tool developed here seems to be highly reliable from *in silico* and *in vitro* results, being sensitive and, theoretically (at least from the current status of reference databases) would not produce false positives from cross-amplification with other European fish species. However, more developments are recommended to completely prevent false positives. Although the BLAST assay only retrieved significant match with the target species, in theory it would be possible to get such cross-amplification with other species still not introduced in the databases. Expanding the current reference databases is necessary for adequate implementation of eDNA methodology for aquatic species detection (Goldberg et al., 2016). On the other hand, false positives may be caused by DNA from dead animals, avian feces, farm discharges or fishing bait (Merkes, McCalla, Jensen, Gaikowski, & Amberg, 2014; Hänfling et al., 2016; Clusa et al., 2017). eDNA may still be detected when the individuals are gone because it is persistent in cold waters (Ficetola et al., 2008). False positives may also be recorded because of contamination during fieldwork or in the laboratory (Thomsen, & Willerslev, 2015). Sampling replications, both temporal and from different places in a river, and the use of good laboratory and field practices, including the use of a blank control sample during fieldwork, will help to solve these problems (Ficetola et al., 2015; Goldberg et al., 2016).

Another important issue to consider when working with eDNA is the possibility of obtaining false negatives from field samples. False negatives may occur in the field for various reasons: when a species is scarce and its DNA has a low concentration (Ficetola et al., 2008); the presence of inhibitors in the sample (Goldberg et al., 2014); or when the activity of a species changes seasonally (De Souza, Godwin, Renshaw, & Larson, 2016). In the case described here, the absence of positive results for the *Ameiurus* sp and *M. salmoides* in the Ebro River could be example of false negatives. It is possible that the number of water sample replicates was insufficient, or that the populations were very scarce. It is also possible that they were not at the sampling points examined, since these species seems to have a preference for reservoirs (Doadrio, 2001), and three of the samples were taken far away from reservoirs.

Despite the problems discussed above, the success of eDNA for detecting populations was confirmed from different studies. Doi et al., (2017) found a relationship between eDNA concentration and fish abundance in Saba river (Japan), where they detected *Plecoglossus altivelis* eDNA from all the places where visual detection was positive, but not when individuals were not found. Adrian-Kalchhauser, & Burkhardt-Holm (2016) successfully detected invasive gobies in Rhine River in Switzerland. These and other examples demonstrate that eDNA methods applied in rivers can cover equal or greater distances than traditional electrofishing (Evans, Shirey, Wieringa, Mahon, & Lamberti, 2017). Notwithstanding, the application of eDNA to monitoring river systems has some intrinsic limitations due to the nature of running waters. Goldberg et al. (2014) suggested that it is not possible to infer a spatial reference in lotic systems from eDNA, because suspended DNA may be transported far away from the population source. Deiner & Altermatt (2014) found eDNA from two target invertebrates 9-12 km downstream from established populations. Other studies have found DNA transport over shorter distances. Civade et al. (2016) showed downstream eDNA transport for only 2-3 km in low flow in the Tier River (France), and Jane et al. (2015) also found that eDNA travel was reduced at low flow. In any case, eDNA can at least give an overview of the biodiversity in a river system (Rius, Bourne, Hornsby, & Chapman, 2015; Deiner, Fronhofer, Mächler, Walser, & Altermatt, 2016). A positive PCR for any of the seven species in this study could be considered a signal of alert, and further investigation in the area, including conventional sampling, would be strongly recommended, because these species are non-native to all Europe (Leppäkoski et al., 2002). Amplicon sequencing to confirm the species would be necessary, as well as physical confirmation of the species occurrence (e.g. from conventional sampling or photographs), before attempting control and management.

Besides early detection, the tool developed here could be useful to monitor the spread of these invasive species (such as checking colonization of upstream dam areas as Yamanaka & Minamoto (2016) did on migratory fishes); for monitoring the efficacy of eradication programs (Davison, Copp, Créach, Vilizzi & Britton, 2017); or in protected areas to avoid disturbing wild populations (Civade et al., 2016). Methods based on eDNA may also be used for monitoring endangered species in their native range, similar to the studies of *Margaritifera margaritifera* (Stoeckle, Kuehn & Geist, 2016; Carlsson et al., 2017), *Lepisosteus oculatus* (Boothroyd, Mandrak, Fox & Wilson, 2016) and *Zearaja maugeana* (Weltz et al., 2017). A possible weakness of this non-quantitative method is that it determines only presence or absence of a species; however, it is easy to apply in routine surveys, since it does not require special technology. It is faster and more economical than metabarcoding (Comtet, Sandionigi, Viard, & Casiraghi, 2015; Taberlet, Coissac, Pompanon, Brochmann, & Willerslev, 2012) or qPCR (Darling & Blum, 2007), since the reagents needed for one sample cost about 12€ in 2017. Bioinformatics analysis is not necessary for interpreting the results, in contrast with Next Generation Sequencing methods such as metabarcoding (Coissac, Riaz, & Puillandre, 2012). The whole process can be completed in one or two days, and it is possible to analyze many samples at the same time.

As a result of the work described here, the set of taxon-specific primers developed is ready for detecting seven of the commonest invasive fish species in Europe directly from water samples, based on environmental DNA, even at very low densities. This powerful and economical method may be directly applied for early detection of all these species in European waters.

Acknowledgements

We would like to thank to Confederación Hidrográfica del Ebro and Joaquín Guerrero from Gobierno de Aragón for the information supplied. We also want to thank Marta Pascual from Centro de Acuicultura Vegas del Guadiana for sending the tissue samples of the invasive species. We are grateful to Natividad Franch Ventura from Parc Natural del Delta de l'Ebre for their collaboration to collect the water sample Ebro point four and five and aquarium sample number five. Finally we would like to thank their collaboration to Carlos Vicente Buitrago and Javier González Sanz from Acuario de Zaragoza for sending the rest of aquarium samples. Laura Clusa holds a PCTI Grant from the Asturias Regional Government, referenced BP14-145. This work was supported by Principality of Asturias, Regional Grant GRUPIN-2014-093, and the EU RIA Contract 689682 – AMBER. The authors have no conflict of interest to declare. We would like to thank the editor and the two anonymous reviewers who helped to improve the manuscript.

Supporting information

Additional Supporting Information may be found online in the supporting information tab for this article:

Table S1. List of non-native fish species present in Europe and countries where they occur, from different databases.

Table S2. List of native and exotic freshwater fish species occurring in the Iberian Peninsula by family.

Table S3. Sequences of the amplicons obtained using species-specific primers from tissue and water samples.

Data Accessibility

Sequences of tissue for 16S rRNA and COI genes are available in GenBank with the accession numbers: KU510486, KU510498, KU510509, and KY231824- KY231850. Sequences of the amplicons obtained using taxa-specific primers from tissue and water samples (both aquarium and Ebro River) are available in DDBJ (DNA Data Bank of Japan) with the accession numbers: LC198795- LC198812 and in supplementary Table S3.

References

Adrian-Kalchhauser, I., & Burkhardt-Holm, P. (2016). An eDNA assay to monitor a globally invasive fish species from flowing freshwater. *PLoS ONE*, 11, 1–22.

Almeida, D., Merino-Aguirre, R., Vilizzi, L., & Copp, G. H. (2014). Interspecific aggressive behaviour of invasive pumpkinseed *Lepomis gibbosus* in Iberian fresh waters. *PLoS ONE*, 9, e88038 http://doi.org/10.1371/journal.pone.0088038

Altschul, S. F., Gish, W., Miller, W., Myers, E. W., & Lipman, D. J. (1990). Basic Local Alignment Search Tool. *Journal of Molecular Biology*, 215, 403–410.

Ardura, A., Zaiko, A., Martinez, J. L., Samulioviene, A., Semenova, A., & Garcia-Vazquez, E. (2015). eDNA and specific primers for early detection of invasive species–a case study on the bivalve *Rangia cuneata*, currently spreading in Europe. *Marine Environmental Research*, 112, 48–55.

554 Blackburn, T. M., Pyšek, P., Bacher, S., Carlton, J. T., Duncan, R. P., Jarošík, V., …
555 Richardson, D. M. (2011). A proposed unified framework for biological
556 invasions. *Trends in Ecology & Evolution*, 26, 333–339.
557 Blanchet, S. (2012). The use of molecular tools in invasion biology: an emphasis on
558 freshwater ecosystems. *Fisheries Management and Ecology*, 19, 120–132.
559 Boothroyd, M., Mandrak, N. E., Fox, M. & Wilson, C. C. (2016). Environmental DNA
560 (eDNA) detection and habitat occupancy of threatened spotted gar (*Lepisosteus*
561 *oculatus*). *Aquatic Conservation Marine and Freshwater Ecosystem*, 26, 1107–
562 1119.
563 Braig, E. C., & Johnson, D. L. (2003). Impact of black bullhead (*Ameiurus melas*) on
564 turbidity in a diked wetland. *Hydrobiologia*, 490, 11–21.
565 Britton, J. R., Davies, G. D., & Brazier, M. (2009). Eradication of the invasive
566 *Pseudorasbora parva* results in increased growth and production of native fishes.
567 *Ecology of Freshwater Fish*, 18, 8–14.
568 Caiola, N., & De Sostoa, A. (2002). First record of the asiatic cyprinid *Pseudorasbora*
569 *parva* in the Iberian Peninsula. *Journal of Fish Biology*, 61, 1058–1060.
570 Carlsson, J. E. L., Egan, D., Collins, P. C., Farrell, E. D., Igoe, F. & Carlsson, J. (2017).
571 A qPCR MGB probe based eDNA assay for European freshwater pearl mussel
572 (*Margaritifera margaritifera* L.). *Aquatic Conservation Marine and Freshwater*
573 *Ecosystem*, 2017, 1–4.
574 Chown, S. L., Hodgins, K. A., Griffin, P. C., Oakeshott, J. G., Byrne, M., & Hoffmann,
575 A. A. (2015). Biological invasions, climate change and genomics. *Evolutionary*
576 *Applications*, 8, 23–46.
577 Civade, R., Dejean, T., Valentini, A., Roset, N., Raymond, J. C., Bonin, A., ... Pont, D.
578 (2016). Spatial representativeness of environmental DNA metabarcoding signal
579 for fish biodiversity assessment in a natural freshwater system. *PLoS One*, 11, 11-
580 19.
581 Clavero, M., & García-Berthou, E. (2005). Invasive species are a leading cause of animal
582 extinctions. *Trends in Ecology and Evolution*, 20, 110.
583 Clusa, L., Ardura, A., Gower, F., Miralles, L., Tsartsianidou, V., Zaiko, A., & Garcia-
584 Vazquez, E. (2016). An easy phylogenetically informative method to trace the
585 globally invasive *Potamopyrgus* mud snail from river's eDNA. *PLoS ONE*, 11,
586 e0162899. http://doi.org/10.1371/journal.pone.0162899
587 Clusa, L., Ardura, A., Fernández, S., Roca, A. A., & García-Vázquez, E. (2017). An
588 extremely sensitive nested PCR-RFLP mitochondrial marker for detection and
589 identification of salmonids in eDNA from water samples. *PeerJ*, 5, e3045.
590 Coissac, E., Riaz, T., & Puillandre, N. (2012). Bioinformatic challenges for DNA
591 metabarcoding of plants and animals. *Molecular Ecology*, 21, 1834–1847.
592 Comtet, T., Sandionigi, A., Viard, F., & Casiraghi, M. (2015). DNA (meta)barcoding of
593 biological invasions: a powerful tool to elucidate invasion processes and help
594 managing aliens. *Biological Invasions*, 17, 905–922.
595 Confederación hidrográfica del Ebro. http://www.chebro.es accessed in November 2016.
596 Copp, G. H., Tarkan, A. S., Masson, G., Godard, M. J., Koščo, J., Kováč, V., …
597 Blackwell, B. G. (2016). A review of growth and life-history traits of native and
598 non-native European populations of black bullhead *Ameiurus melas*. *Reviews in*
599 *Fish Biology and Fisheries*, 26, 441–469.
600 Darling, J. A., & Blum, M. J. (2007). DNA-based methods for monitoring invasive
601 species: A review and prospectus. *Biological Invasions*, 9, 751–765.

Davison, P. I., Copp, G. H., Créach V., Vilizzi L. & Britton J. R. (2017). Application of environmental DNA analysis to inform invasive fish eradication operations. *The Science of Nature*, 104, 1-35.

Davison, P. I., Créach, V., Liang, W.-J., Andreou, D., Britton, J. R., & Copp, G. H. (2016). Laboratory and field validation of a simple method for detecting four species of non-native freshwater fish using eDNA. *Journal of Fish Biology*, 44, 1–12.

Davy, C. M., Kidd, A. G., & Wilson, C. C. (2015). Development and validation of environmental DNA (eDNA) markers for detection of freshwater turtles. *PLoS One*, 10, e0130965

Deiner, K., & Altermatt, F. (2014). Transport distance of invertebrate environmental DNA in a natural river. *PLoS ONE*, 9, e88786. http://doi.org/10.1371/journal.pone.0088786

Deiner, K., Fronhofer, E. A., Mächler, E., Walser, J. C., & Altermatt, F. (2016). Environmental DNA reveals that rivers are conveyer belts of biodiversity information. *Nature Communications*, 7, 12544. http://doi.org/10.1038/ncomms12544

De Souza, L. S., Godwin, J. C., Renshaw, M. A., & Larson, E. (2016). Environmental DNA (eDNA) detection probability is influenced by seasonal activity of organisms. *PLoS One*, 11, 1–15

Devloo-Delva, F., Miralles, L., Ardura, A., Borrell, Y. J., Pejovic, I., Tsartsianidou, V., & Garcia-Vazquez, E. (2016). Detection and characterisation of the biopollutant *Xenostrobus securis* (Lamarck 1819) Asturian population from DNA Barcoding and eBarcoding. Marine Pollution *Bulletin*, 105, 23–29.

Doadrio. (2001). Atlas y Libro Rojo de los Peces Continentales de España. Dirección General de Conservación de la Naturaleza. Ministerio de Medio Ambiente, Madrid p364. ISBN: 84-8014-313-4

Doi, H., Inui, R., Akamatsu, Y., Kanno, K., Yamanaka, H., Takahara, T., & Minamoto T. (2017). Environmental DNA analysis for estimating the abundance and biomass of stream fish. *Freshwater Biology*, 62, 30–39.

Elvira, B., & Almodóvar, A. (2001). Freshwater fish introductions in Spain : facts and figures at the beginning of the 21st century. *Journal of Fish Biology*, 59, 323–331.

Estoup, A., Largiader, C. R., Perrot, E., & Chourrout, D. (1996). Rapid one-tube DNA extraction for reliable PCR detection of fish polymorphic markers and transgenes. *Molecular Marine Biology and Biotechnology*, 5, 295–298.

Evans, N. T., Shirey, P. D., Wieringa, J. G., Mahon, A. R., & Lamberti, G. A. (2017). Comparative cost and effort of fish distribution detection via environmental DNA analysis and electrofishing. *Fisheries*, 42, 90–99.

Ficetola, G. F., Miaud, C., Pompanon, F., & Taberlet, P. (2008). Species detection using environmental DNA from water samples. *Biology Letters*, 4, 423–425.

Ficetola, G. F., Pansu, J., Bonin, A., Coissac, E., Giguet-Covex, C., De Barba, M., ... Taberlet, P. (2015). Replication levels, false presences and the estimation of the presence/absence from eDNA metabarcoding data. *Molecular Ecology Resources*, 15, 543–556.

Foote, A.D., Thomsen, P.F., Sveegaard, S., Wahlberg, M., Kielgast, J., Kyhn, L.A., … Gilbert, M.T.P. (2012). Investigating the potential use of environmental DNA (eDNA) for genetic monitoring of marine mammals. *PloS One*, 7, e41781.

Freyhof, J., & Brooks, E. (2011). European Red List of Freshwater fishes. Luxembourg: Publications Office of the European Union. http://doi.org/10.2779/85903

Furlan, E. M., & Gleeson, D. (2016). Environmental DNA detection of redfin perch, *Perca fluviatilis*. *Conservation Genetics Resources*, 8, 115–118.

García-Berthou, E., Alcaraz, C., Pou-Rovira, Q., Zamora, L., Coenders, G., & Feo, C. (2005). Introduction pathways and establishment rates of invasive aquatic species in Europe. *Canadian Journal of Fisheries and Aquatic Sciences*, 62, 453–463.

Geller, J., Meyer, C., Parker, M., & Hawk, H. (2013). Redesign of PCR primers for mitochondrial cytochrome c oxidase subunit I for marine invertebrates and application in all-taxa biotic surveys. *Molecular Ecology Resources*, 13, 851–861.

Goldberg, C. S., Strickler, K. M., & Pilliod, D. S. (2014). Moving environmental DNA methods from concept to practice for monitoring aquatic macroorganisms. *Biological Conservation*, 183, 1–3.

Goldberg, C. S., Turner, C. R., Deiner, K., Klymus, K. E., Thomsen, P.F., Murphy, M. A., ... Taberlet, P. (2016). Critical considerations for the application of environmental DNA methods to detect aquatic species. *Methods in Ecology and Evolution*, 7, 1299–307.

Gozlan, R. E., Andreou, D., Asaeda, T., Beyer, K., Bouhadad, R., Burnard, D., … Britton, J. R. (2010). Pan-continental invasion of *Pseudorasbora parva*: Towards a better understanding of freshwater fish invasions. *Fish and Fisheries*, 11, 315–340.

Gozlan, R. E., St-Hilaire, S., Feist, S. W., Martin, P. & Kent, M. L. (2005). Disease threat to European fish. *Nature*, 435, 1046.

Gustavson, M. S., Collins, P. C., Finarelli, J. A., Egan, D., Whelan, K., Carlsson, J. E. L., & Carlsson, J. (2015). An eDNA assay for Irish *Petromyzon marinus* and *Salmo trutta* and field validation in running water. *Journal of Fish Biology*, 87, 1254–1262.

Hall, T. (1999). BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *Nucleic Acids Symposium Series*, 41, 95–98.

Hänfling, B., Handley, L. L., Read, D. S., Hahn, C., Li, J., Nichols, P., ... Winfield, I. J. (2016). Environmental DNA metabarcoding of lake fish communities reflects long-term data from established survey methods. *Molecular Ecology*, 25, 3101–3119.

Havel, J. E., Kovalenko, K. E., Thomaz, S. M., Amalfitano, S., & Kats, L. B. (2015). Aquatic invasive species: challenges for the future. *Hydrobiologia*, 750, 147–170.

Hurlbert, S. H., & Mulla, M. S. (1981). Inpacts of mosquitofish (*Gambusia affinis*) predation on plankton communities. *Hydrobiologia*, 83, 121–151.

Hurlbert, S. H., Zedler, J., & Fairbanks, D. (1972). Ecosystem alteration by mosquitofish (*Gambusia affinis*) predation. *Science*, 175, 639–641.

Jane, S. F., Wilcox, T. M., Mckelvey, K. S., Young, M. K., Schwartz, M. K., Lowe, W. H., ... Whiteley, A. R. (2015). Distance, flow and PCR inhibition: eDNA dynamics in two headwater streams. *Molecular Ecology Resources*, 15, 216–227.

Jiang, J., Alderisio, K. A., Singh, A., & Xiao, L. (2005). Development of procedures for direct extraction of *Cryptosporidium* DNA from water concentrates and for relief of PCR inhibitors. *Applied and Environmental Microbiology*, 71, 1135–1141.

Keskin, E. (2014). Detection of invasive freshwater fish species using environmental DNA survey. *Biochemical Systematics and Ecology*, 56, 68–74.

Leppäkoski, E., Gollasch, S., & Olenin, S. (2002). *Invasive aquatic species of Europe. Distribution, impacts and management*. Kluwer Academic Publishers: Dordrecht, The Netherlands. ISBN 978-90-481-6111-9 DOI 10.1007/978-94-015-9956-6

699 Leuven, R. S. E. W., van der Velde, G., Baijens, I., Snijders, J., van der Zwart, C.,
700 Lenders, H. J. R., & bij de Vaate, A. (2009). The river Rhine: A global highway
701 for dispersal of aquatic invasive species. *Biological Invasions*, 11, 1989–2008.
702 Maceda-Veiga, A. (2013). Towards the conservation of freshwater fish: Iberian Rivers as
703 an example of threats and management practices. *Reviews in Fish Biology and*
704 *Fisheries*, 23, 1-22.
705 Maceda-Veiga, A., Escribano-Alacid, J., de Sostoa, A., & García-Berthou, E. (2013). The
706 aquarium trade as a potential source of fish introductions in southwestern Europe.
707 *Biological Invasions*, 15, 2707–2716.
708 Machida, R. J., Leray, M., Ho, S-L., & Knowlton, N. (2017). Metazoan mitochondrial
709 gene sequence reference datasets for taxonomic assignment of environmental
710 samples. *Scientific Data* 4, 170027, doi: 10.1038/sdata.2017.27
711 Maezono, Y., & Miyashita, T. (2002). Community-level impacts induced by introduced
712 largemouth bass and bluegill in farm ponds in Japan. *Biological Conservation*,
713 109, 111–121.
714 Maretto, F., Reffo, E., Dalvit, C., Barcaccia, G., & Mantovani, R. (2007). Finding 16S
715 rRNA gene-based SNPs for the genetic traceability of commercial species
716 belonging to Gadiformes. *Italian Journal of Animal Science* ,6, 161–163.
717 Merkes, C. M., McCalla, S. G., Jensen, N. R., Gaikowski, M.P., & Amberg, J. J. (2014).
718 Persistence of DNA in carcasses, slime and avian feces may affect interpretation
719 of environmental DNA data. *PLoS One*, 9, e113346.
720 Ministerio de Medio Ambiente. (2007). La invasión de Especies exóticas en los ríos.
721 Madrid.
722 Palumbi, S. R., Martin, A., Romano, S., McMillan, W. O., Stice, L., & Grabowski, G.
723 (2002). The simple fool's guide to PCR. version 2.0. Department of Zoology and
724 Kewalo Marine Laboratory. pp. 28, University of Hawaii, Honolulu.
725 Piaggio, A. J., Engeman, R. M., Hopken, M. W., Humphrey, J. S., Keacher, K. L., Bruce,
726 W. E., & Avery, M. L. (2014). Detecting an elusive invasive species: a diagnostic
727 PCR to detect Burmese python in Florida waters and an assessment of persistence
728 of environmental DNA. Molecular Ecology Resources, 14, 374–380
729 Pilliod, D.S., Goldberg, C.S., Arkle, R.S., & Waits, L.P. (2014). Factors influencing
730 detection of eDNA from a stream-dwelling amphibian. Molecular Ecology
731 Resources, 14, 109–116.
732 Pinder, A. C., Gozlan, R. E., & Britton, J. R. (2005). Dispersal of the invasive topmouth
733 gudgeon, *Pseudorasbora parva* in the UK: A vector for an emergent infectious
734 disease. *Fisheries Management and Ecology*, 12, 411–414.
735 Pyke, G. H. (2008). Plague minnow or mosquito fish? A review of the biology and
736 impacts of introduced Gambusia species. *Annual Review of Ecology, Evolution,*
737 *and Systematics*, 39, 171–191.
738 Regional Government of Aragón webpage:
739 http://www.aragon.es/estaticos/GobiernoAragon/Departamentos/AgriculturaGan
740 aderiaMedioAmbiente/TEMAS_MEDIO_AMBIENTE/AREAS/BIODIVERSID
741 AD/01_Especies_Exoticas_Invasoras/PECES.pdf accessed in October 2017.
742 Remon, J., Bower, D. S., Gaston, T. F., Clulow, J., & Mahony, M. J. (2016). Stable
743 isotope analyses reveal predation on amphibians by a globally invasive fish
744 (*Gambusia holbrooki*). *Aquatic Conservation: Marine and Freshwater*
745 *Ecosystems*, 26, 724–735.
746 Ribeiro, F., & Leunda, P. M. (2012). Non-native fish impacts on Mediterranean
747 freshwater ecosystems: Current knowledge and research needs. *Fisheries*
748 *Management and Ecology*, 19, 142–156.

Rius, M., Bourne, S., Hornsby, H. G., & Chapman, M. A. (2015). Applications of next-generation sequencing to the study of biological invasions. *Current Zoology*, 61, 488–504.

Rozas, J., Sánchez-DelBarrio, J. C., Messeguer, X., & Rozas, R. (2003). DnaSP, DNA polymorphism analyses by the coalescent and other methods. *Bioinformatics*, 19, 2496–2497.

Saitou, N., & Nei, M. (1987). The neighbour-joining method: a new method for reconstructing phylogenetic trees. *Molecularl Biology and Evolution*, 4, 406–425.

Sanz, N., Araguas, R. M., Vidal, O., Diez-del-Molino, D., Fernández-Cebrián, R., & García-Marín, J. L. (2013). Genetic characterization of the invasive mosquitofish (*Gambusia* spp.) introduced to Europe: Population structure and colonization routes. *Biological Invasions*, 15, 2333–2346.

Savini, D., Occhipinti-Ambrogi, A., Marchini, A., Tricarico, E., Gherardi, F., Olenin, S., & Gollasch, S. (2010). The top 27 animal alien species introduced into Europe for aquaculture and related activities. *Journal of Applied Ichthyology*, 26, 1–7.

Simon, A., Gozlan, R. E., Britton, J. R., van Oosterhout, C., & Hänfling, B. (2014). Human induced stepping-stone colonisation of an admixed founder population: the spread of topmouth gudgeon (*Pseudorasbora parva*) in Europe. *Aquatic Sciences*, 77, 17–25.

Sterud, E., & Jørgensen, A. (2006). Pumpkinseed *Lepomis gibbosus* (Linnaeus, 1758) (Centrarchidae) and associated parasites introduced to Norway. *Aquatic Invasions*, 1, 278–280.

Stoeckle, B. C., Kuehn, R. & Geist, J. (2016). Environmental DNA as a monitoring tool for the endangered freshwater pearl mussel (*Margaritifera margaritifera* L.): a substitute for classical monitoring approaches? *Aquatic Conservation: Marine and Freshwater Ecosystem*, 26,1120–1129.

Taberlet, P., Coissac, E., Pompanon, F., Brochmann, C., & Willerslev, E. (2012). Towards next-generation biodiversity assessment using DNA metabarcoding. *Molecular Ecology*, 21, 2045–2050.

Takahara, T., Minamoto, T., & Doi, H. (2013). Using environmental DNA to estimate the distribution of an invasive fish species in ponds. *PLoS ONE*, 8, e56584. http://doi.org/10.1371/journal.pone.0056584

Tamura, K., Nei, M., & Kumar, S. (2004). Prospects for inferring very large phylogenies by using the neighbor-joining method. *PNAS*, 101, 11030–11035.

Tamura, K., Stecher, G., Peterson, D., Filipski, A., & Kumar, S. (2013). MEGA6 : Molecular Evolutionary Genetics Analysis version 6.0. *Molecular Biology and Evolution*, 30, 2725-2729.

Thompson, J. D., Higgins, D. G., & Gibson, T. J. (1994). CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Research*, 22, 4673–4680.

Thomsen, P. F., Kielgast, J., Iversen, L. L., Møller, P. R., Rasmussen, M., & Willerslev, E. (2012). Detection of a diverse marine fish fauna using environmental DNA from seawater samples. *PLoS ONE*, 7, 1–9.

Thomsen, P. F. & Willerslev, E. (2015). Environmental DNA - An emerging tool in conservation for monitoring past and present biodiversity. *Biological Conservation* , 183, 4–18

Tsunoda, H., Mitsuo, Y., Ohira, M., Doi, M., & Senga, Y. (2010). Change of fish fauna in ponds after eradication of invasive piscivorous largemouth bass, *Micropterus*

salmoides, in north-eastern Japan. *Aquatic Conservation: Marine and Freshwater Ecosystems*, 20, 710–716.

Turner, C. R., Barnes, M. A., Xu, C. C. Y., Jones, S. E., Jerde, C. L., & Lodge, D. M. (2014). Particle size distribution and optimal capture of aqueous macrobial eDNA. *Methods in Ecology and Evolution*, 5, 676–684.

Uchii, K., Doi, H., & Minamoto, T. (2016). A novel environmental DNA approach to quantify the cryptic invasion of non-native *genotypes*. *Molecular Ecology Resources*, 16, 415–422.

Ushio, M., Fukuda, H., Inoue, T., Makoto, K., Kishida, O., Sato, K., … Miya, M. (2017). Environmental DNA enables detection of terrestrial mammals from forest pond water. *Molecular Ecology Resources*, doi: 10.1111/1755-0998.12690

Vences, M., Lyra, M. L., Perl, R. G. B., Bletz, M. C., Stanković, D., Lopes, C. M., ... Steinfartz, S. (2016). Freshwater vertebrate metabarcoding on Illumina platforms using double-indexed primers of the mitochondrial 16S rRNA gene. *Conservation Genetic Resources*, 8, 323–327.

Weyl, O. L. F., & Lewis, H. (2016). First record of predation by the alien invasive freshwater fish *Micropterus salmoides* L . (Centrarchidae) on migrating estuarine fishes in South Africa. *African Zoology Journal*, 41, 294–296.

Yamanaka, H., & Minamoto, T. (2016). The use of environmental DNA of fishes as an efficient method of determining habitat connectivity. *Ecological Indicators*, 62, 147–153.

Weltz, K., Lyle, J. M., Ovenden, J., Morgan, J. A. T., Moreno, D. A. & Semmens, J. M.(2017) Application of environmental DNA to detect an endangered marine skate species in the wild. *PLoS One* 12, 1–16