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RESEARCH ARTICLE

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

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## Abstract

1. Biological invasions are a global threat to biodiversity, and many arise from deliberate introductions.
2. The American freshwater fish *Micropterus salmoides* and *Ameiurus* spp. (*Ameiurus melas* and *Ameiurus nebulosus*) were introduced to Europe for recreational fishing, *Gambusia holbrooki* and *Gambusia affinis* were introduced for mosquito population control, and *Lepomis gibbosus* was introduced as an ornamental species. The Asiatic *Pseudorasbora parva* was acquired inadvertently as an accompanying species in fish consignments.
3. This article presents a novel approach for detecting these species directly from water samples based on a panel of five taxon-specific primers within *16S* rDNA.
4. The primers were validated from tissue, in aquarium experiments, and from Ebro River water samples (Spain). With a simple polymerase chain reaction (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 0.89–100 pg $mL^{-1}$.
5. This sensitive and economical tool can be used to control European invasions of these species and to preserve native biodiversity.

**KEYWORDS**

*16S* rDNA, alien species, biological control, eDNA, fish, PCR, species-specific primers

## 1 | INTRODUCTION

Biological invasions are an important threat to biodiversity (Chown et al., 2015), as they often result in local extinctions or extirpation of native species (Clavero & García-Berthou, 2005). Aquatic species are translocated worldwide for various purposes, ranging 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. (*Ameiurus melas* and *Ameiurus nebulosus*) were introduced into many European waters for recreational fishing (Copp et al., 2016; Savini et al., 2010). The mosquitofish *Gambusia holbrooki* and *Gambusia affinis* were widely introduced to Europe for mosquito control (Pyke, 2008) from their native range in North America (Sanz et al., 2013). Another source of the spread of exotic fish is from imports for the 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 (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 behaviour 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 through predation, sometimes causing their decline or extinction (Maezono & Miyashita, 2002; Weyl & Lewis, 2016). *Ameiurus* spp. 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* spp.) 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), that are capable of transmission to native fish species (Gozlan, St-Hilaire, Feist, Martin, & Kent, 2005).

The 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 the eradication of *M. salmoides* (Tsunoda, Mitsuo, Ohira, Doi, & Senga, 2010). In one lake of the Lake District in the north west of the UK, populations of native *Rutilus rutilus* and *Abramis brama* increased after *P. parva* eradication (Britton, Davies, & Brazier, 2009). The 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 excretions and sloughed cells, mucus excretion, and defaecation (Goldberg, Strickler, & Pilliod, 2014). This method seems to be sensitive and efficient, and unlike most classic sampling methods (electrofishing, netting) 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; Clusa et al., 2016; Devloo-Delva et al., 2016), fishes (Adrian-Kalchhauser & Burkhardt-Holm, 2016; Furlan & Gleeson, 2016; Gustavson et al., 2015; Takahara, Minamoto, & Doi, 2013; Uchii, Doi, & Minamoto, 2016), amphibians (Ficetola et al., 2008; Pilliod, Goldberg, Arkle, & Waits, 2014), reptiles (Davy, Kidd, & Wilson, 2015; Piaggio et al., 2014), 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 the early detection of seven common invasive fish species: *A. melas*, *A. nebulosus*, *G. affinis*, *G. holbrooki*, *L. gibbosus*, *M. salmoides*, and *P. parva*, from water samples. If successful, the method could be used by managers for river surveillance. For this purpose, new specific primers were developed and tested experimentally *in vitro* and *in aquaria*, as well as from field water samples. These 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/act.php?id=BOE-A-2013-8565). They all occur in the Ebro River (north-east Spain), as reported in the webpage of the Regional Government of Aragón (http://www.aragon.es/estaticos/GobiernoAragon/Departamentos/AgriculturaGanaderiaMedioAmbiente/TEMAS_MEDIO_AMBIENTE/AREAS/BIODIVERSIDAD/01_Especies_Exoticas_Invasoras/PECES.pdf); thus, the new tool was field tested in Ebro River waters. The method could also be applied in other European waters for the surveillance of these invasive alien species.

## 2 | MATERIALS AND METHODS

### 2.1 | Species studied

For confirming the adequacy of the choice of species, an exhaustive search was performed in three databases of invasive species: EASIN (European Alien Species Information Network; https://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 International Union for Conservation of Nature (IUCN). The criterion of choice was species invasiveness to European countries. The species selected were known to be invasive in a large number of European countries (the top five). A list of non-native species invasive to Europe was compiled (Table S1), and five of the taxa chosen for this study (*Ameiurus* sp., *Gambusia* sp., *L. gibbosus*, *M. salmoides*, and *P. parva*) are listed there among the most common 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).

### 2.2 | Design of species-specific primers

The method applied is based on conventional polymerase chain reaction (PCR). The *16S* rRNA gene was chosen for the design of the primers based on reference nucleotide sequences from GenBank (http://www.ncbi.nlm.nih.gov), together with 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 related species, especially in fishes (Maretto, Reffo, Dalvit, Barcaccia, & Mantovani, 2007; Vences et al., 2016). The number of sequences for fishes in databases is similar to other mitochondrial genes such as cytochrome *c* oxidase subunit 1 (*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 analysed with DNASP (Rozas, Sánchez-DelBarrio, Messeguer, & Rozas, 2003). The different haplotypes were visualized using 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 the reference species collected, were located. These regions were used to design a set of specific primers (Table 1).

**TABLE 1** Taxon-specific primers designed for this study

| Species | Primer | Sequence (5′–3′) | Annealing temperature | [$Mg^{2+}$] | Amplicon size | Stock | Last dilution | Detection limit |
|---|---|---|---|---|---|---|---|---|
| *Gambusia* spp. | Ga-16S-F<br>Ga-16S-R | GRAACCAACTGACCCCTGCTT<br>GTTTTGTGAGCTGCGGCTCTWTA | 68°C | 1 mM | 117 bp | 0.535 μg $mL^{-1}$ | 1/600 000 | 0.89 pg $mL^{-1}$ |
| *Micropterus salmoides* | MiSa-16S-F<br>MiSa-16S-R | WCATCCCRAAACAAAGGGCY<br>AATTCTGTTCATTAGAGCGGAGG | 68°C | 2 mM | 142 bp | 0.57 μg $mL^{-1}$ | 1/100 000 | 5.7 pg $mL^{-1}$ |
| *Ameiurus* spp. | Am-16S-F<br>Am-16S-R | CGTCAAGAACYCAGTTRAACT<br>GWTTCTGYGACTTAGAGTTGTCA | 65°C | 1 mM | 134 bp | 0.7 μg $mL^{-1}$ | 1/5000 | 140 pg $mL^{-1}$ |
| *Pseudorasbora parva* | PsPa-16S-F<br>PsPa-16S-R | GTTTAAYCATGTTAAACAACTTAT<br>TTCGTTGATCGACTATGTGT | 58°C | 2.5 mM | 192 bp | 0.5 μg $mL^{-1}$ | 1/5000 | 100 pg $mL^{-1}$ |
| *Lepomis gibbosus* | LeGi-16S-F<br>LeGi-16S-R | GGACACGGGGCTAAACCAAAT<br>GGGCTCTTAGTTGTGGAATTGCA | 68°C | 1 mM | 113 bp | 0.535 μg $mL^{-1}$ | 1/600 000 | 0.89 pg $mL^{-1}$ |

Primer sequences, annealing temperature, $Mg^2$ concentration, expected amplicon size (in base pairs), initial DNA concentration used for testing the sensitivity of the primer pairs (stock), maximum dilution (last dilution), and corresponding DNA concentration (detection limit) for which it is possible to obtain a polymerase chain reaction (PCR) product visible in agarose gel with the primer pairs in the conditions assayed.

## 2.3 | Fish tissue and water sampling in the field and in the aquarium

Tissue samples were provided by Centro de Acuicultura Vegas del Guadiana. Genetic barcoding was performed using *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, 1 L of water was collected in sterile plastic bottles from each sampling point, both in the aquariums and in the 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 min between filtrations. Filters were placed individually within 15-mL tubes and stored at −20°C until DNA extraction.

## 2.4 | DNA extraction and PCR conditions

DNA from tissue samples was extracted using Chelex resin, as described by Estoup, Largiader, Perrot, and Chourrout (1996). DNA from water samples was extracted directly from the filters with the PowerWater® DNA Isolation Kit (Mobio Laboratories), following the manufacturer's recommendations. The eDNA extractions were performed under sterile conditions, in a laboratory unit where there were no other tissue samples, and 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), 1 L 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, and 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). To confirm that cross-amplification, negative results were not linked 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 20 μL, including full-strength Green GoTaq® Buffer, $MgCl_2$, 0.25 mM deoxyribonucleotide triphosphates (dNTPs), 1 μM of each primer, 2 μL of template DNA, and 0.65U of DNA Taq polymerase (Promega). The PCR conditions were as follows: an initial denaturation step at 95°C for 5 min, 35 cycles at 94°C for 30 s, annealing at the temperature of choice for 30 s, and elongation at 72°C for 30 s. A final step of elongation was set at 72°C for 10 min. 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 2.5 μL 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 6 μL of DNA template. Bovine serum albumin (BSA; 200 ng $mL^{-1}$) was added to 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.

## 2.5 | *In silico* and *in vitro* validation of the designed primers

The new taxon-specific primers were tested first *in silico* with the BLAST tool on the NCBI webpage (Altschul, Gish, Miller, Myers, & Lipman, 1990) to confirm that they aligned significantly with just 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), and thus native species of such families do not occur in Spanish waters and false positives are not expected. There are Iberian native species from the Cyprinidae, however, but not from the same genus considered in this study (*Pseudorasbora*), as well as other exotic cyprinid genera (Table S2). Thus, for cross-amplification two native cyprinids (*Phoxinus phoxinus* and *Squalius pyrenaicus*) and two non-native cyprinids (*Carassius auratus* and *Leuciscus idus*), as well as 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 (*A. melas*, *G. holbrooki*, *L. gibosus*, *M. salmoides*, and *P. 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.

## 2.6 | *In situ* validation of the designed primers

The method was also validated in environmental DNA from controlled aquarium water samples provided by Zaragoza Aquarium and the Ebro Delta Ecomuseum. Water samples (1 L) from tanks containing individuals of each of the taxa studied and other fish were analysed 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 the Ebro River as a case study. The seven species have been reported at several sites in the Ebro basin (Ministerio de Medio Ambiente, 2007). At 930 km, it is the second longest river in the Iberian Peninsula with the second highest flow rate, with an average discharge of 400 $m^3 s^{-1}$ (information retrieved from Confederación Hidrográfica del Ebro webpage; http://www.chebro.es).

In December 2015, water samples were taken from five points along the Ebro River. Three of them were sampled from running waters, far away from reservoirs, and two were sampled 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 downstream in Movera (sampling point 3). The two samples collected in the Ebro River delta were sampling points 4 and 5 in two ponds, where Caiola and De Sostoa (2002) reported the occurrence of *P. parva*.

Two replicate 1-L water samples were collected with sterile bottles from each sampling point, placing the bottle as close to the substrate as

**TABLE 2** Aquarium experiments

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

Water volume in the aquaria (in L), number and size of individuals of the target species, and other species present in each aquarium together with the target species (number of individuals in parenthesis).

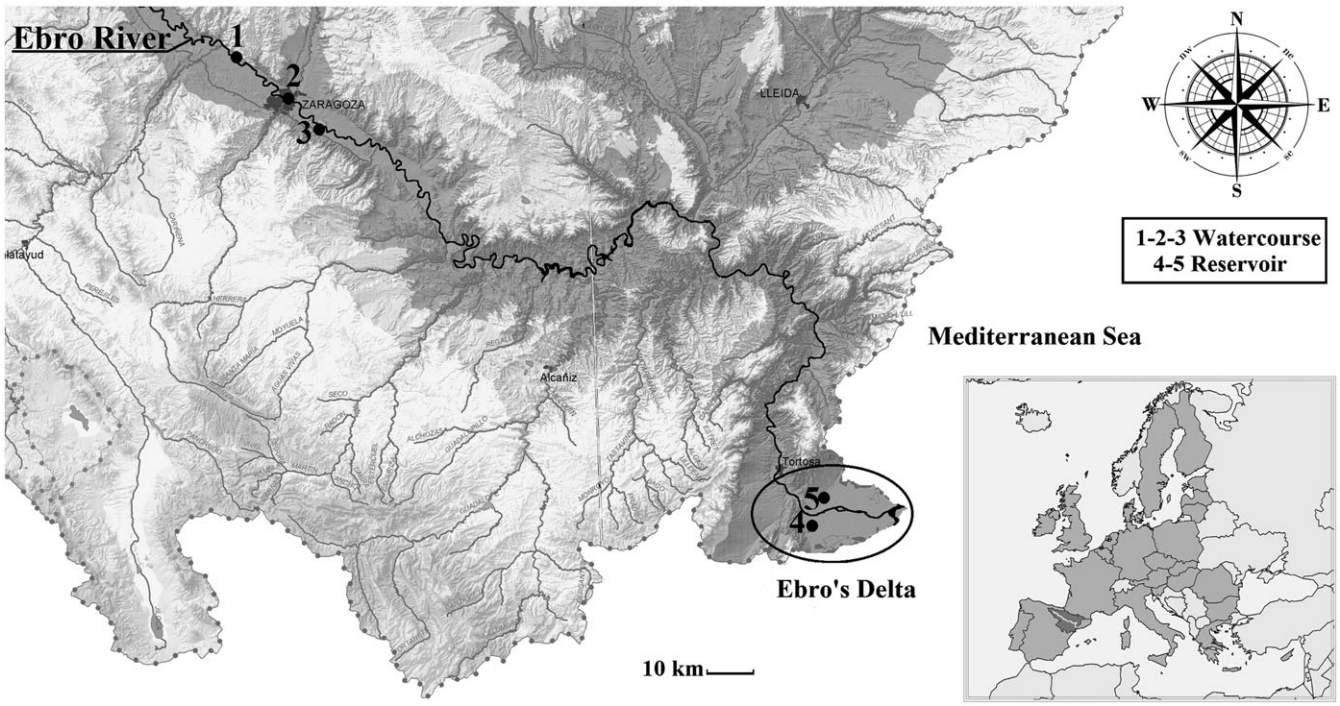


**FIGURE 1** Map of the Ebro River and its location in the Iberian Peninsula. The Ebro River is highlighted in black and the five sampling points are shown (downloaded from Confederación Hidrográfica del Ebro, http://www.chebro.es)

possible. They were immediately transported to the laboratory on ice and then frozen. At sampling point 1, a survey was carried out along the riverside using a landing net. In total, 100 m was surveyed from the riverside. At the rest of the locations manual netting was not possible because of very high river flow rates and rapid currents.

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

### 2.7 | 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 performed to check whether the primers could distinguish between different species of a genus. Different reference sequences of *Ameiurus bruneus*, *Ameiurus catus*, *Ameiurus melas*, *Ameiurus natalis*, *Ameiurus nebulosus*, *Gambusia affinis*, and *Gambusia holbrooki* were downloaded from GenBank. The sequences obtained in this study as well as the additional reference sequences were aligned with CLUSTALW 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 10 000 bootstraps. The evolutionary distances were computed using the maximum composite likelihood method (Tamura, Nei, & Kumar, 2004).

## 3 | RESULTS

### 3.1 | Design of specific primers and experimental validation

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

From *in silico* BLAST assays, the new primers retrieved significant alignments only with the species for which they were designed. Consistent with these results, cross-amplification was not found for the assayed species, and for each pair of primers positive PCR amplification occurred only from the 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 the *16S* rRNA gene with universal primers (Palumbi et al., 2002) was found for all of 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 *16S* rRNA genes of each tissue sample are available in GenBank (accession numbers KU510486, KU510498, KU510509, and KY231824–KY231835).

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

For 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 DNA Data Bank of Japan accession numbers LC198795–LC198812). The negative controls for extraction (*Nc1* in Figure 3) were clean, and any contamination in the process could be disregarded.

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 the *Ameiurus* species (*A. brunneus*, *A. catus*, and *A. natalis*) that are exotic to Europe but not considered to be invasive (Figure 4a for *Gambusia*; Figure 4b for *Ameiurus*).

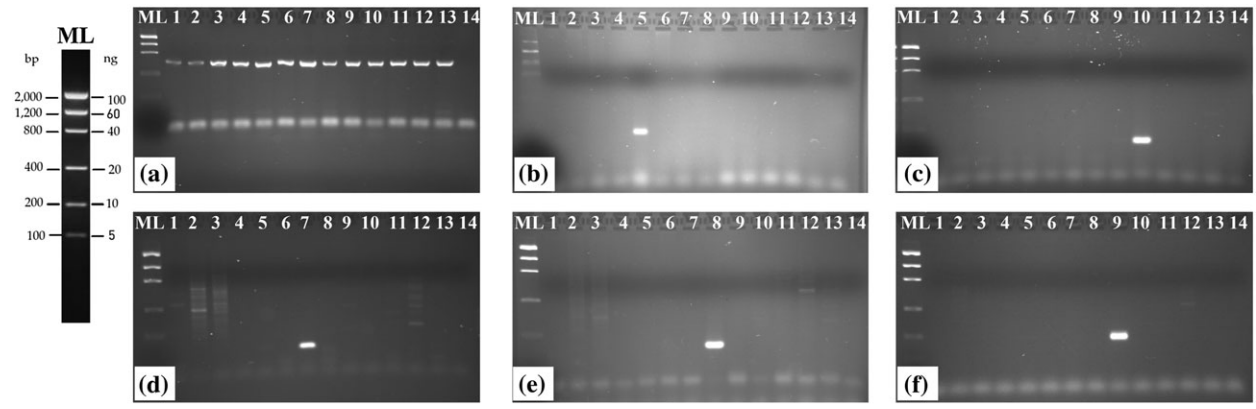


**FIGURE 2** Agarose gels (2%) showing the results of cross-amplification experiments for each specific marker. *16S* rDNA polymerase chain reaction (PCR) amplified with: (a) universal primers (Palumbi et al., 2002); (b) specific primers for *Ameiurus* spp.; (c) *Gambusia* spp.; (d) *Lepomis gibbosus*; (e) *Micropterus salmoides*; (f) *Pseudorasbora parva*. Lanes: 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

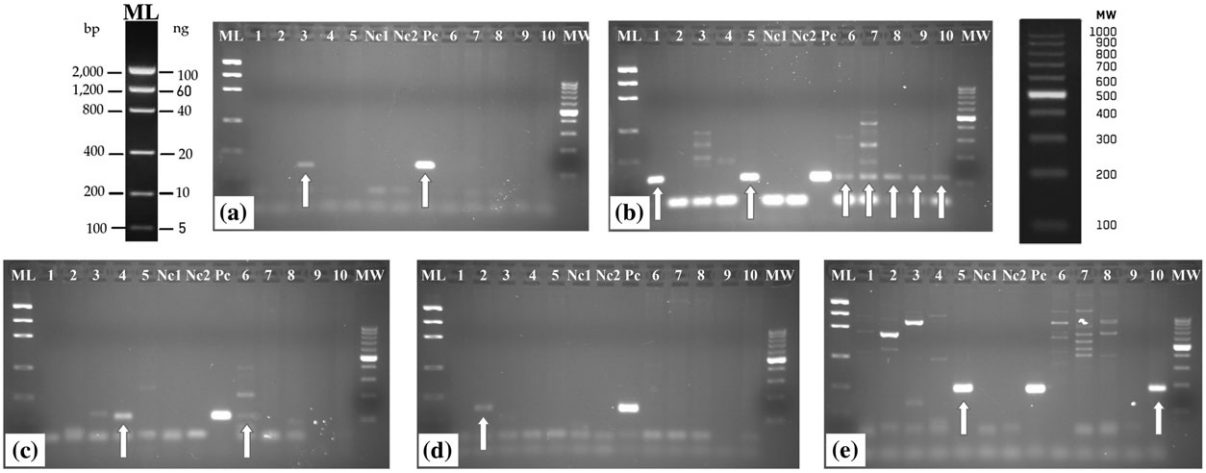


**FIGURE 3** Agarose gels (2%) showing polymerase chain reaction (PCR) products from environmental DNA (eDNA) for each specific marker: (a) *Ameiurus* spp.; (b) *Gambusia* spp.; (c) *Lepomis gibbosus*; (d) *Micropterus salmoides*; (e) *Pseudorasbora parva*. Lanes: ladder (ML); 1, aquarium 1; 2, aquarium 2; 3, aquarium 3; 4, aquarium 4; 5, aquarium 5; 6, Ebro River sampling point 1; 7, Ebro River sampling point 2; 8, Ebro River sampling point 3; 9, Ebro River sampling point 4; 10, Ebro River sampling point 5. Negative controls: Nc1, negative control for extraction; Nc2, negative control for PCR. Pc: positive control with tissue DNA of each species

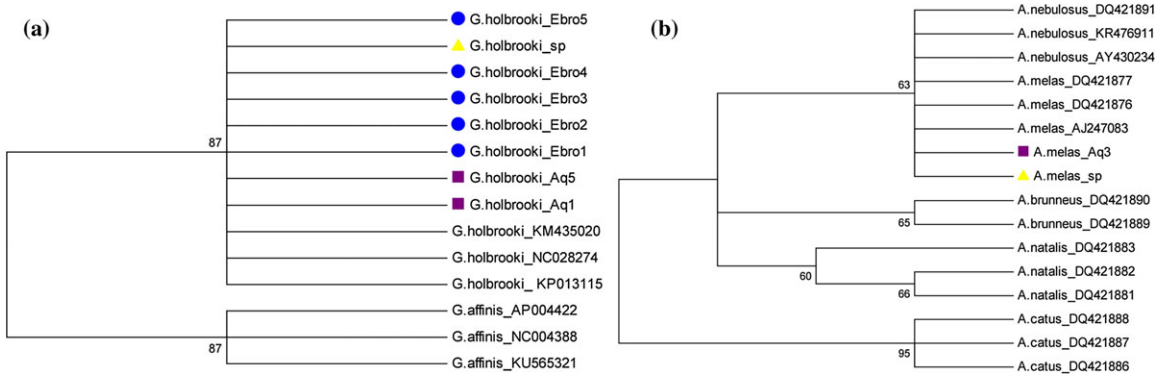


**FIGURE 4** Phylogenetic trees reconstructed from sequences obtained in this work and references from GenBank (with accession number indicated). Tissue-positive samples are indicated with a yellow triangle, aquarium samples are indicated with a purple square, and Ebro River samples are indicated with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

**TABLE 3** Ebro River field environmental DNA (eDNA) results

| Sampling points | Coordinates | *Ameiurus* spp. | *Gambusia* spp. | *Lepomis gibbosus* | *Micropterus salmoides* | *Pseudorasbora parva* |
|---|---|---|---|---|---|---|
| 1 | 41.736952°N, −0.992233°W | – | + | + | – | – |
| 2 | 41.658574°N, −0.878066°W | – | + | – | – | – |
| 3 | 41.632217°N, −0.837865°W | – | + | – | – | – |
| 4 | 40.64336°N, 0.7104704°E | – | + | – | – | – |
| 5 | 40.72397°N, 0.721833°E | – | + | – | – | + |

Sampling points along the Ebro River, and their coordinates, and polymerase chain reaction (PCR) amplification results obtained with the taxon-specific primers designed in this study: +, positive PCR; –, negative PCR.

## 3.2 | Assays in field water samples: Ebro River

The results from the Ebro River (Figure 3; Table 3) revealed DNA of three of the target taxa from the water samples analysed: *Gambusia* spp., *L. gibbosus*, and *P. parva*. Positive detection was obtained in the two replicates of eDNA samples taken. *Gambusia* spp. were found from all of the sampling points. *Lepomis gibbosus* was found from sampling point 1 and *P. parva* was found from sampling point 5 in the Ebro River delta, where it had been reported by Caiola and De Sostoa (2002). For *Ameiurus* spp. 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, and 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 the eDNA analysis for detecting this species from running waters.

## 4 | DISCUSSION

The set of specific primers designed and validated in this study has proved very sensitive for the detection of seven of the most common 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 (with 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 to river water samples. This study contains several innovations. This is the first time that primers have been validated for detecting *L. gibbosus* from running water samples, the first time that primers have been designed within the *16S* rDNA gene for *P. parva*, and this is the first eDNA method at all, to our knowledge, for the other five species (*Gambusia* spp., *Ameiurus* spp., and *M. salmoides*).

Finding positive amplification results from running water in the Ebro River was encouraging because it confirms the power of the eDNA-based methodology. Despite the high flow and rapid currents 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 (0.2 μm) can recover eDNA quantities from small water volumes (similar to the ones used in this study), equivalent to those obtained from the filtration of larger water volumes through larger pore filters. Other studies have used water samples ranging from 250 mL up to 5 L (Goldberg et al., 2016). The *Gambusia* primers enabled the detection of a *G. holbrooki* population in a zone (sampling points 1–3) where the river is wide (133 ± 24 m), 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 showed 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 primers specific to *L. gibbosus* and *P. parva*. Therefore, the method would be useful for detecting these species in the early invasion stages, when the population size is still low and might be overlooked by traditional sampling methods. Owing to its sensitivity, the method could be applied to detect these seven invasive species in other European streams where invasion is suspected. It could be especially useful in large rivers, such as the Rhine, which is connected to nearly all of the large rivers in southwestern, southern, central, and eastern Europe, and could be the entrance point for 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 matches with the target species, in theory it would be possible to get such cross-amplification with other species not yet included in the databases. Expanding the current reference databases is necessary for the 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 faeces, farm discharge, or fishing bait (Clusa et al., 2017; Hänfling et al., 2016; Merkes, McCalla, Jensen, Gaikowski, & Amberg, 2014). eDNA may still be detected when the individuals are gone because it persists 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: if a species is scarce the associated eDNA will be present in low concentrations (Ficetola et al., 2008); inhibitors could be present in the sample (Goldberg et al., 2014); or the activity of a species might change seasonally (De Souza, Godwin, Renshaw, & Larson, 2016). In the case described here, the absence of positive results for the *Ameiurus* spp. and *M. salmoides* in the Ebro River could be an 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, as these species seem 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 has been confirmed in different studies. Doi et al. (2017) found a relationship between eDNA concentration and fish abundance in the Saba River (Japan), where they detected *Plecoglossus altivelis* eDNA from all of the sites where visual detection was positive, but not when individuals were not found. Adrian-Kalchhauser and Burkhardt-Holm (2016) successfully detected invasive gobies in the River Rhine 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). Nevertheless, the application of eDNA for monitoring river systems has some intrinsic limitations because of 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 and Altermatt (2014) found eDNA from two target invertebrate species 9–12 km downstream from established populations. Other studies have found DNA transport over shorter distances. Civade et al. (2016) found downstream eDNA transport of only 2–3 km in low flow in the Tier River (France), and Jane et al. (2015) also found that eDNA transport was reduced in low flows. In any case, eDNA can at least give an overview of the biodiversity in a river system (Deiner, Fronhofer, Mächler, Walser, & Altermatt, 2016; Rius, Bourne, Hornsby, & Chapman, 2015). 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 of 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 for monitoring the spread of these invasive species (such as checking colonization of upstream dam areas, as Yamanaka and Minamoto (2016) did for migratory fishes), for monitoring the efficacy of eradication programmes (Davison, Copp, Créach, Vilizzi, & Britton, 2017), or for monitoring 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* (Carlsson et al., 2017; Stoeckle, Kuehn, & Geist, 2016), *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 the presence or absence of a species; however, it is easy to apply in routine surveys because 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 quantitative PCR (Darling & Blum, 2007), as 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 1 or 2 days, and it is possible to analyse 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 most common invasive fish species in Europe directly from water samples, based on eDNA, even at very low densities. This powerful and economical method may be directly applied for early detection of all of these species in European waters.

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## SUPPORTING INFORMATION

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

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