Title:

Citizen warnings and post checkout molecular confirmations using eDNA as a combined strategy for updating invasive species distributions.

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Abstract

Citizen warnings alerting about the presence of invasive species has been claimed as fundamental in the strategies addressed to manage aquatic invasions. A great volume of online information coming from fishermen and ordinary citizens, about detection of new species in their communities is currently available. Unfortunately, it is not always implemented as a useful tool by agencies, and within stakeholders protocols, to prevent and manage biological invasions. In this work, we have used as a case study the wels catfish *Silurus glanis* Linnaeus, 1758. Online blogs, webpages, videos, magazines, and newspapers, were searched for news about the presence and spread of *S. glanis* in the Iberian Peninsula until June 2016. This information was compiled with official reports (scientific papers and regional and national governmental reports) to create a map showing the putative current pattern of *S. glanis* invasion through Iberian freshwater ecosystems. The current situation pointed to *Silurus*'s presence in six of the seven main river basins of the Iberian territory. Since non-official reports need post-alerts confirmations, we have also set up a molecular pilot study designing genus specific primers to detect this species in environmental DNA (eDNA) from the reported locations. In the pilot study, primers were tested on eDNA samples extracted from experimental aquariums and on real environmental samples taken from different basins in Spain (Ebro, Douro and Tagus). In all these basins *S. glanis* was detected. Official S*ilurus* reports were confirmed with two molecular markers in five out of the six cases (83%) assayed in this work, and in two out of three non-official reports (66%) coming from fishermen websites and newspaper reports. The proposed combined strategy (citizen alerts and the eDNA detection method) can be a helpful tool in early detection of invasive species allowing fast and effective management actions by stakeholders.

Keywords: *Silurus glanis*, invasive species; Spain; Portugal; environmental DNA; management; catfish; wels.

Introduction

The species *Silurus glanis* Linnaeus, 1758, also known as wels, catfish or sheatfish, is the largest freshwater fish in Europe and, together with *Silurus aristotelis* Garman, 1890, represent the *Silurus* genus in Europe (Copp et al., 2009; Triantafyllidis, Abatzopoulos & Economidis, 1999; Triantafyllidis, John, Leonardos & Guyomard, 2002). The catfish was originally introduced into the Iberian Peninsula in 1974 through the Segre River (Ebro basin) by Roland Lorkowsky (a German biologist) and soon after that at the Mequinenza-Ribarroja and in Flix Reservoirs (Carol, 2007; Doadrio, 2001). After this initial spread, it was also reported in the Tagus drainage during the first decade of this century (Doadrio, 2001; Pérez-Bote & Roso, 2009). Since then, the anglers have rumored about the spread of catfish in different areas of Tagus river basin (Pérez-Bote & Roso, 2011). Introductions were reported later in the Catalonia coastal basins at Llobregat River and Sau-Susqueda Reservoirs (Benejam, Carol, Benito & García-Berthou, 2007; Carol, Benejam, Pou-Rovira, Zamora & García-Berthou, 2003); it has also been recently reported in the Guadalquivir River (Alegre & Ceballos, 2006; Moreno-Valcárcel, Miguel, & Fernández-Delgado, 2013). The spread of alien invasive fishes does not respect political boundaries and in 2015 *S. glanis* reached Portugal through downstream movement along the Tagus River from populations in Spain (Gkenas, Gago, Mesquita & Alves, 2015).

The *S. glanis* species is robust enough to easily resist its transport, allowing its translocation to other areas outside its native range. It is difficult to catch it using traditional methods such as nets or electric angling (Pérez-Bote & Roso, 2011). In addition, the fish can be discovered years after the introduction, when the population have reached high densities (Freire, Genzano,

Neumann-Leitão & Pérez, 2014; Adrian-Kalchhauser & Burkhardt-Holm 2016); this can cause difficulties for management plans or eradication attempts (Adrian-Kalchhauser & Burkhardt-Holm, 2016). Early alerts about the presence of this species are fundamental. Unfortunately, scientific reports often seem to be “out of date” and do not go as fast as needed (Banha, Ilhéu, & Anastácio, 2015). Moreover, there is not an efficient and quick "add and check" mechanism, by which governmental agencies, after receiving reports from fishermen or other citizens, could give a clear and updated picture about the real distribution pattern of this invasive species. The Iberian Peninsula shows great levels of endemism and native fishes have evolved without the presence of native piscivores (Clavero, Blanco-Garrido, & Prenda, 2004; Copp et al., 2009; Crivelli, 1995; Doadrio, 2001). Therefore, the impact of *S. glanis* on Iberian freshwaters can be much greater than in the case of other European countries. This makes urgent the need for rigorous and exhaustive species assessments for early detection of this dangerous invasive species.

The use of PCR has made a breakthrough in species identification with regard to traditional morphological identification (Taberlet, Coissac, Pompanon, Brochmann, & Willerslev, 2012). Nowadays, it is possible to design species-specific primers for rapid detection of a species of interest within the community even when the DNA is partially degraded. Thus, it is possible to increase the accuracy and reduce the cost and time required (Ficetola, Miaud, Pompanon & Taberlet, 2008; Farrington et al., 2015). Environmental DNA (eDNA) assays have been used recently for revealing the presence of species owing to the organism's vestigial particles that remain in the environment without the need to catch the organism (e.g. the environmental eDNA calibration study of the invasive Asian carp in USA; Baerwaldt et al., 2014). It requires less time, equipment, man-power, skills and financial resources than the traditional monitoring methods such as electrofishing, angling or diving (Rees, Maddison, Middleditch,

89 Patmore & Gough, 2014). According to Bohmann et al. (2014) and Adrian-Kalchhauser &
90 Burkhardt-Holm (2016), even untrained people can collect samples, and the assay can be
91 accomplished on a simple thermocycler.

92

93 Two main aims of this study are: (i) to update the presumptive current situation of the
94 invasive wels catfish *S. glanis* in Iberian freshwater ecosystems using formal and non-formal
95 reports; and, (ii) to design and test specific primers for *S. glanis* that allow its use as a simple
96 molecular tool to confirm *S. glanis* presence in water samples using the eDNA from rivers,
97 lakes and artificial ponds. This combined strategy would be useful for efficient detection,
98 prevention and management policies for this invasive fish, including invasions at an early
99 stage, and helping stakeholders in taking effective and fast management actions.

100

101 **Material and methods**

102

103 *An update S. glanis database for the Iberian Peninsula*

104 The inland territory of Spain is politically divided in 15 communities from which all, except
105 the Principality of Asturias, are crossed by seven main rivers/watersheds (Ebro, Douro, Tagus,
106 Guadiana, Guadalquivir, Jucar and Segura). The continental territory of Portugal is divided in
107 18 districts that are all crossed by, at least, one of the main five river basins (Tagus, Douro,
108 Minho, Guadiana and Mondego). Thus, four out of five Portuguese main river basins are
109 shared with Spain. An updated *S. glanis* database was created to compile all the relevant
110 information about its spreading pattern across Spain and Portugal (Appendix 1). For that
111 purpose, 15 blogs, 6 web-pages, videos, more than 30 magazines and newspapers, 13

scientific papers and all the official information from the Ministry of Agriculture, Food and Environment of Spain about the spread and presence of *S. glanis* in the Iberian Peninsula until June 2016 were consulted, scrutinized and summarized (Appendix 1). The sources of information were classified as official only when coming from scientific papers and governmental reports, while the rest were designated as "unofficial". A *Silurus* spreading map representing all the information included in this database was designed using QGIS 2.14 Essen.

In silico S. glanis's specific primer design

Cytochrome oxidase sub-unit I (COI) and *16S* sequences from public databases as Genbank and BOLD from *Silurus sp.* were downloaded for designing *S. glanis* specific primers. All the sequences were aligned using ClustalW application on BioEdit (Hall, 1999). Specific primers were designed using two different softwares: Prise2 (PCR Primer Design Software) (Huang, Yang, Chrobak, & Borneman, 2014); and, Primer-BLAST (Ye et al., 2012). The following parameters were selected in both software programs following software recommendations: A primer length range between 18 and 28 base pairs (bp); a PCR product size between 200 and 400 bp; a melting temperature (Tm) between 52 and 70ºC with a maximum difference of 2ºC between forward and reverse primers and finally a percentage of GC between 25 and 75%. Different settings for the 3' end in Prise2 were tested and those which showed the best results (100% similarity in target sequences and the lowest % in non-target species) were selected (a 2.1.0 design). For the case of primer design with Primer-BLAST, specific primers that have at least two mismatches within five bases from the 3' end of the primer were chosen.

In vitro assays: Tissue samples, DNA extractions, PCR conditions and *Primer specificity tests*

Fourteen tissue samples of *Silurus* sp. were obtained from the Zoological Research Museum Alexander Koenig (Bonn, Germany). DNA was extracted with the QIAGEN QIAamp DNA Mini Kit (Tissue Protocol) following the manufacturer instructions and stored at -20ºC. All the individuals were barcoded using the *Cytochrome oxidase* subunit I (COI) gene (Ward, Zemlak, Innes, Last, & Hebert, 2005). Genetic identifications were done using the BOLD system identification engine (http://www.boldsystems.org/index.php/IDS_OpenIdEngine) and species identifications were accepted only with more than 98% of identity.

The specific primers developed here were used to obtain specific amplicons in *Silurus's* tissue samples through PCR procedures in a 2720 Thermal Cycler, Applied Biosystems. For a final volume of 20 µL, Green Go Taq Flexi Buffer (1X) PROMEGA®, $MgCl_2$ (from 1 mM to 2.5 mM depending of each primer pair), dNTPs (0.5 mM), 0.2 µM of each primer, 0.5 U/µL of Go Taq G2 Flexi Polymerase PROMEGA®, H_2O and 0.5 µL of isolated DNA were used. The PCR program included an initial activation step of 95ºC during 5 min, followed by 35 cycles with a denaturation step at 95ºC during 30 s, 30 s of annealing between 65ºC to 70ºC (depending of each primer pair) and an extension step at 72ºC during 30 s. Finally, the PCR cycling end with a final elongation at 72ºC for 7 min. PCR products were checked on a 2% agarose gel stained with SimplySafe™.

Fish tissue samples from eight other fishes that usually share their habitat with *Silurus sp.* in Spain (*Alburnus alburnus* (Linnaeus, 1758)*; Scardinius erythrophthalmus* (Linnaeus, 1758)*; Squalius pyrenaicus* (Günther, 1868)*; Leuciscus idus (*Linnaeus, 1758)*; Phoxinus phoxinus* (Linnaeus, 1758)*; Pseudorasbora parva* (Temminck & Schlegel, 1846)*; Carassius auratus* (Linnaeus, 1758)*; Ameiurus melas* (Rafinesque, 1820) (Elvira & Almodóvar, 2001), were

used for testing primers specificity tests following the already described PCR procedures for the newly specific primers developed in this work. Primers pairs revealing unspecific amplification patterns were discarded for further analysis.

In situ assays using environmental DNA from artificial lab positive controls and Iberian river basins

In order to create an artificial lab positive control of *S. glanis* eDNA in water, an already dead and frozen *S. glanis* fish (size: 49 cm; weight 654 g) was provided by a fisherman from Loire (Blois, France). After *COI* barcoding and genetic identification, 1g portion of muscle from this fish were put for 7 days in 500 ml of distilled water. Additionally, the Aquarium of Zaragoza provided us with two water samples (1 liter each one) taken from two different tanks. The first tank sample (C) was taken from an exhibition tank in the Aquarium where *Silurus glanis* cohabits with 48 different species (Appendix 2). The second one (SZ2) was taken from a tank with a total volume of 13,000 l where one individual of *Silurus glanis* was isolated in quarantine.

Freshwater samples were obtained from areas with, and without, reported presence of *S. glanis* (1.5 liter each). Samples from the Ebro (Spain) and Tagus rivers (Spain), together with the Ullibarri-Gamboa reservoir (in Vitoria, Basque Country, Spain) and one sample from the river Loire (France) were tested and used as positive controls since all of them have been officially cited as locations with *S. glanis* presence (Table 1). Freshwater samples from the Nora River and from San Andrés de los Tacones reservoir (Asturias, Spain) as well as one seawater sample from the Port of Gijón were used as negative controls (there are no reports about presence of *S. glanis* in those locations) (Table 1). Freshwater samples without any

official information about the presence of *Silurus* sp. but where fishermen have already reported *Silurus* sp. were also tested: Ricobayo dam (Zamora, Spain); San Martin de la Vega pond (Madrid, Spain); and, Aldeanueva del Codonal pond (Segovia, Spain) (Table 1).

Water samples were taken in sterile plastic bottles at 75 cm below the surface. The coordinates of each sampling point were recorded and the bottles were properly labeled and stored in cold ice for transportation to the laboratory, where they were immediately frozen (-20 Celsius degrees) until the filtering process. The water samples (1.5 l) were filtered with a vacuum pump through a Supor® PES Membrane Disc Filters with a pore size of 0.2µm and a diameter of 47mm for DNA extraction using a commercial kit (PowerWater® DNA isolation kit) from MO-BIO Laboratories, Inc. (Carlsbad, California, USA) and following manufacturer's recommendations. Moreover, DNA extractions were conducted using negative controls and in different days for samples using sterility measures inside laminar air flow chamber. All lab equipment were continuously disinfected by UV light, absolute ethanol and 10% bleach solution cleanings to prevent contamination. After that, the lab utensils were rinsed with distilled water and autoclaved. The eDNA quantity was checked in 2% agarose gels stained with SimplySafe™.

The PCR's were conducted in a 2720 Thermal Cycler, Applied Biosystems. In essence they were similar to those already described in the PCR section (see above) but 200 ng/µL of Bovine Serum Albumin (BSA) and 4 µl of eDNA template were used this time following Jiang, Alderisio, Singh, & Xiao (2005) recommendations when working with eDNA from natural freshwater samples. The PCR program was just slightly modified increasing the number of the three step cycling (55 times) (Takahara, Minamoto, & Doi, 2015; Thomsen et

al., 2012, Mauvisseau et al. 2017). In order to avoid false positives and negatives several PCR replicates of each sample were done. PCR products were checked on a 2% agarose gel stained with SimplySafe™. Positive results when working with DNA samples were recorded as such only when a unique band appeared, showing the same expected sizes as those registered for control samples.

In addition, all PCR bands obtained were purified using the GeneMATRIX Agarose-Out DNA Purification Kit (EurX®) and were sent to Macrogen Europe for sequencing. The sequences obtained were BLASTed within the National Center for Biotechnology Information for confirming species identification.

This study has been approved by the Committee of Ethics of the Principality of Asturias, with the reference 100/16 for GRUPIN-2014-093.

Results

The current picture of the catfish S. glanis invasion process in the Iberian Peninsula using official and non-official reports

The Figure 1 summarizes the evolution of invasive process of the species *S. glanis* in the Iberian Peninsula since the first release of 32 young individuals in 1974 in the Segre River (Ebro basin, Spain) (Figure 1a) (Carol, 2007) until nowadays (2016) (Figure 1b, 1c and 1d). The Figure 1b shows a slow catfish spread process occurred over a period of 25 years across the Ebro basin. At the beginning only Catalonia was affected by the *Silurus* invasion, but at the end of the year 2000, the *S. glanis* presence was already officially reported at four out of

231 the 14 Spanish communities (≈ 29%): Catalonia, Aragon (Doadrio, 2001; Elvira & Almodóvar,
232 2001); Navarre (Navarra, 1997); and, Basque Country (Asensio, Pinedo, & Markina, 1995).
233 The possibility of its presence in La Rioja was also commented in an official report (Zaldívar,
234 1994) which could increase the percentage of affected communities to 36% of the inland
235 Spanish territory.

236

237 A dramatic expansion of the *Silurus* invasion process is evident for the last 16 years (Figure
238 1c and 1d). The species was officially recorded in nine Spanish communities (64%) from year
239 2000 to 2010. There were several new presence reports out of the Ebro basin in Catalonia
240 (Aparicio & Julià, 2009; Benejam et al., 2007; Carol et al., 2003); it was also reported in
241 Castile-La Mancha (Nicola, Noriega, Gómez, & Martín, 2009), Extremadura (Doadrio, 2001;
242 Pérez-Bote & Roso, 2009), Andalusia (Alegre & Ceballos, 2006) and Valencia (Comunitat
243 Valenciana, 2009, 2010). Moreover, non-official reports increase this occupation percentage
244 to 11 of the Spanish communities (78%) adding presence registers in Madrid (Plataforma
245 Jarama Vivo, 2001) and Castile and Leon (PescaLeón, 2010). Additionally, Greenpeace
246 reported in 2006 the first announcement of *Silurus* presence in Portugal, specifically in the
247 Vale do Guadiana Natural Park (Greenpeace, 2006). At the end of 2010, four of the seven
248 main river Spanish basins were officially reported as invaded by *S. glanis* (Ebro, Tagus,
249 Guadalquivir and Jucar). Moreover, it was also non-officially reported by fishermen in four
250 different reservoirs of the Douro watershed (Spain) and in the Guadiana river basin (Portugal)
251 (Figure 1c).

252

253 Figure 1d shows a summary of the *S. glanis* presence from all the official and non-official
254 reports done between 2011 and 2016. Until now, there are no reports about *S. glanis* in Murcia

(the Segura watershed), Cantabria (origin of the Ebro river) or in Galicia (influenced by the Duero river). However, *S. glanis* is still in expansion with new official reports in many other regions within Castile and Leon, especially in the area surrounding the city of Soria (Diario de Soria, 2014; El Norte de Castilla, 2015c; Junta de Castilla y León, 2015; Tardajos de Duero, 2015). There was also a second official report in the Guadalquivir River (Moreno-Valcárcel et al., 2013) and its presence in the Sitjar reservoir in Castellon (Valencia) was also officially reported (Levante-EMV, 2012). The first official report of *Silurus* in Portugal in the Tagus watershed was announced in 2015 (Gkenas et al., 2015). In addition, there have been more non-official reports in the last five years within the communities of Castile and Leon (Hay Pesca!, 2012; Ieltxu Vega, 2011), Extremadura (Navalmoral Digital, 2015) and Madrid (Ediciones El País, 2016; Hay Pesca!, 2014) (Figure 1d). Taking into account both, official and non-official reports, the current picture about this biological invasion seems to be that nowadays six out of the seven main river basins in Spain (86%) and two out of the five main watersheds in Portugal (40%) have been invaded by *S. glanis*. The only exception is the Segura watershed where no official or unofficial report has alerted about *S. glanis* presence.

In silico development of a molecular specific tool for confirmation and early detection of S. glanis

Four different primer pairs were designed in this work for specific detection of *S. glanis* in the eDNA. First finding in this work was related with the low levels of inter specific genetic variation in Silurids found in the two genes under study. No software assays were able to find species-specific primer pairs for either the *COI* gene nor for the *16S* gene. Despite this, the *Primer-BLAST* software gave as result *16S* primer pairs although they were not only useful for the intended target (*S. glanis*) but also for unintended amplicons coming from *Silurus asotus* Linnaeus, 1758, *Silurus biwaensis* (Tomoda, 1961), *Silurus lanzhouensis* Chen, 1977 and

Silurus meridionalis Chen, 1977. This primer pair (**silPB16s**) was considered as a genus specific *16S* marker (Table 2). The *16S* primers designed using *Prise2* (**silPS16s**) could potentially amplify the 100% of target species (*S. glanis*) (Table 2) and just a 3.8% of non-target siluriformes species not native to Iberian Peninsula. The primer design results from Primer-BLAST for the *COI* gene (Table 2) were similar to those previously achieved with the *16S* gene since they were just genus specific (**silPBCOI**). They work well with *S. glanis* (100%) and in other *Silurus* spp. The primer designs using *Prise2* revealed a pair of genus specific primers (**silPSCOI**) that amplify the 100% of the target species (*S. glanis*) and only a 0.5% of non-target species, being that 0.5% *S. aristotelis* (Table 2).

In vitro tests of the molecular genus-specific tools for detecting Silurus

Genetic identifications of the fourteen tissue samples of *Silurus* sp. obtained from the Zoological Research Museum Alexander Koenig (Bonn, Germany) confirmed, with more than 99% of identity, that the tissue samples were from six *S. aristotelis* and eight *S. glanis* individuals. *In vitro* PCR assays on those tissues demonstrated that the four primer pairs produced the expected PCR amplicons (expected sizes: *COI* gene: 150 bp and *16S* gene: 219 bp) and besides this, they did not show any unspecific band pattern (Figure 2). Additional analysis were done in this work to prove the absence of PCR artifacts, or cross-amplifications, with available DNA from sympatric fish species (Figure 3). There were not relevant cross-amplifications with other fishes sharing the *S. glanis* habitat in the case of the primer pairs obtained from the *Prise 2* software (**silPS16s**, **silPSCOI**) (Figure 3). However, that was not the case for the **silPB16s** primers (Figure 3). After this result, primer pairs from the *Primer-BLAST* software were discarded for the upcoming eDNA assays.

In situ tests of the molecular genus-specific tools for detecting Silurus

Primers pairs developed in this work (**silPS16s**, **silPSCOI**) were assayed on *S. glanis* tissue (A), on eDNA from the the artificial lab eDNA positive control (B) and the aquarium of Zaragoza eDNA sample (C) yielding a unique band at the expected sizes (Figure 4). Sequencing of those bands and NCBI Blast procedures showed identities over 96% with the species *S. glanis* (Table 3). Three basins officially reported with *Silurus* fish presence were also tested in this work (Table 1, Figure 4). Two out of the four eDNA samples coming from the Ebro watershed showed **silPS16s** and **silPSCOI** PCR results similar to the positive control assayed in this work (Sample 1.2 (Zaragoza city) and 1.3 (Ullibarri-Gamboa, Vitoria) (Figure 4). The other two samples (1.1 (Utebo, Zaragoza) and 1.4 (Nanclares-Gamboa, Vitoria)) yielded smaller PCR fragments (silPS16s) or negative results (sample 1.4, silPSCOI) (Figure 4). The three eDNA samples from the Tagus basin (2.1 (Villarreal de San Carlos, Caceres), 2.2 (Serradilla, Caceres), 2.3 (San Martin de la Vega, Madrid)) all yielded positive results with both markers that were similar in shape and size to the control patterns (Figure 4). The sample 3.1 (Ricobayo, Zamora) from the Douro basin yielded again positive results however the sample 3.2 (Aldeanueva del Codonal, Segovia) from the same basin gave PCR amplicons that were different to the expected ones (Figure 4). The eDNA Loire sample (Beaugency, France) yielded positive results with the two markers under study (Figure 4). PCR products were sequenced and blasted in the NCBI database and showed identities (>97%) with the species *S. glanis* (Table 3).

The global level of correspondence among the expected (officially or non-officially reported locations of *Silurus* invasion) and the observed detection in the eDNAs samples of *Silurus* spp. assayed in this work (using the new markers developed for the 16S and COI genes) was estimated as 70% (**silPS16s**: 7 out of 10 assayed eDNA samples) and 77% (**silPSCOI**: 7 out

of 9 eDNA assayed samples) (Table 1). Official *Silurus* reports were confirmed with both markers in five out of six cases (83%) and in two out of three (66%) non-official reports coming from fishermen websites and newspaper reports (Table 1).

Discussion

An updated picture about the presence of an invasive species cannot be obtained using only official publications. Administration and governmental reports are rarely updated and show sometimes inexact data (i.e. the current official Spanish list on invasive species (BOE, 2013) is far from being a complete and updated list). In addition, scientific publications often also show an outdated picture due to delays related with manuscript preparations and long review and publication processes. The final result is that most often, alerts on invasive species occur when populations are established, years after the first introduction event (Freire et al., 2014, Adrian-Kalchhauser & Burkhardt-Holm 2016). It seems that the presence of *S. glanis* in the Iberian Peninsula have had a considerable increase, especially in the last 15 years (Figure 1). Cambray (2003) claimed that these exotic game fish species are spreading as a consequence of two key factors; on one hand by anglers and on the other hand by engineering structures as interbasin transfers. Spain has the largest number of dams per km of channel in Europe and also several inter-basin transfers, the Tagus-Segura being the most important of all of them (Vidal-Abarca & Suárez, 2013). On the other side, increasing interest by anglers for new species and in angling opportunities where none existed previously might thus favor invasive species' expansion and the establishment of self-sustained populations (Elvira & Almodóvar, 2001). This could be the result of tourism associated with fishing guide services, an already important economic activity in some reservoirs of the Ebro Basin attracting tourists from

353 Central Europe (Banha, Diniz & Anastacio, 2017) and an emerging activity in some reservoirs
354 from the Tagus basin.

355 This work develops not only the first updated Silurus's map including official and non-official
356 reports but also the first molecular markers for the detection of *Silurus* in eDNA from
357 freshwater ecosystems of the Iberian Peninsula, independently of the stage of the introduction.
358 This is a less expensive technique compared to the traditional monitoring with nets or
359 electrofishing, and thus can be a useful tool since *Silurus* are difficult to catch even with
360 traditional fishing techniques (Pérez-Bote & Roso, 2011). The new molecular markers
361 developed in this work are genus-specific markers that will allow the detection of silurids in
362 Iberian freshwater ecosystems. Their lack of species specificity for the *S. glanis* species will
363 not be a big issue since in Spain *Silurus* sp. is not a native genus. Previous studies have also
364 failed in the attempt to develop species-specific molecular markers (i.e. Japanese salamanders
365 (Fukumoto, Ushimaru, & Minamoto, 2015). Moreover, we set up a pilot study testing these
366 new molecular tools at least in some locations of the main river basins from the Iberian
367 Peninsula. Official S*ilurus* reports were confirmed with two molecular markers in five out of
368 the six cases (83%) assayed in this work, and in two out of three non-officials reports (66%)
369 coming from fishermen websites and newspaper reports.

370

371 Despite high sensitivities, the eDNA assays are susceptible to false positives and false
372 negatives. False positives (error type I) can be explained as eDNA detections when the species
373 of interest is not present. False negatives (error type II) appear when eDNA is not detected
374 being the target species present in the sample (Ficetola et al., 2015). Figure 4 shows that
375 samples coming from the Ebro basin (the starting initial introduction location for *Silurus*)
376 were recorded as a positive for *Silurus* using both molecular markers but the exception of the
377 sample 1.4 (Nanclares de Gamboa, Vitoria, Ebro basin). It would be possible to think that this

can be a false negative. Samples were collected in different points of the same reservoir where the *Silurus* was reported in 1993 (Asensio et al., 1995). False negatives can be caused in some environmental samples by leaf litter, current flow or sampling depth (Bohmann et al., 2014; Darling & Mahon, 2011; Ficetola et al., 2015; Hunter et al., 2015; Jane et al., 2015). Adrian-Kalchhauser & Burkhardt-Holm (2016) have argued that sampling in shallow waters may result in false negatives because DNA concentration would be higher in bottom layers. Besides this, use of replicates is necessary when working with eDNA as argued by Ficetola et al. (2015). Obviously, fishes are not homogeneously distributed in a reservoir which implies fish densities will be heterogeneous compromising the accuracy of the eDNA detection. Despite this, different sampling points from the same reservoir (as it has been done in this work) will help to avoid false negatives. Contamination and/or false positives are usually a serious concern in detection studies (Goldberg et al., 2015). Alarming attitudes after detection could imply the use of economic resources and the establishment of measures to fight against, or control, a biological invasion. In this work, there was no evidence of contamination events and the use of artificial, or natural, positive and negative samples used here worked well and gave no doubts about incurring false detection. Evidences of cross-amplification with other fish species were not found (not even in an aquarium tank with several fish species since amplified band resulted to be just *S. glanis* after sequencing). Good procedures focusing on accurate calibration and avoiding contaminations are essential to consider eDNA as an efficient detection method (Baerwaldt et al., 2014).

New reports about the spread of *S. glanis* in the Iberian Peninsula have been appearing in non-official channels and there is really a need of fast actions to confirm, or discard, the presence of *Silurus* (i.e. Almendra, Ricobayo, Porma and Riano reservoirs (PescaLeón, 2010)). Banha et al. (2015) reasoned that the information and reports published on angling forums are not always rigorous but can be useful for planning field samplings. We observed that in the

403 sample 3.2 assayed here (Aldeanueva del Codonal, Douro basin) “millions of catfishes” were
404 reported in a non-official report (El Norte de Castilla, 2015a). Later they resulted to be
405 *Ameiurus melas* (Order Siluriformes) following press reports, which could be an
406 understandable mistake since for an untrained eye, this species, can be easily confused with *S.*
407 *glanis (El Norte de Castilla, 2015a, 2015b)*. Figure 3 shows that *A. melas* was one of the
408 species that were used to test the primers’s specificities here without apparent cross
409 amplification. The positive results obtained there by our molecular system (Figure 4), and
410 post-checkouts using sequencing (Table 3), indeed indicated the presence of *S. glanis* in this
411 location. Thus, by using these molecular markers controversial reports can be solved or at
412 least could indicate the need for more research in these locations. A different example is the
413 Ricobayo reservoir (Douro basin) used in this work (sample 3.1, Figure 4) which showed
414 *Silurus* presence with the two new molecular markers developed here even when this was not
415 officially reported before. The molecular confirmation add this location as a “positive”
416 together with the official report of the presence of *Silurus* in the Douro river in the
417 surroundings of Soria (Diario de Soria, 2014; El Norte de Castilla, 2015c; Heraldo de Soria,
418 2015; Junta de Castilla y León, 2015). Management measures by Spanish and Portuguese
419 authorities with the aim to avoid the spread of *S. glanis* along the entire river are urgently
420 needed.

421

422 In summary, a combination of comprehensive reviews of citizens and institutions online alerts
423 and post molecular checkouts using genus-specific molecular tools can be proposed as a rapid,
424 cheap and an indeed affordable strategy. Upcoming developments in this strategy should be to
425 focus in the designs of Taqman probes to convert this qualitative test to a quantitative one
426 (qPCRs) and in increasing the number of locations sampled within river basins to develop a

more complete field study. This could be a first step to more complete informative phases in management plans dealing with dangerous invasive species.

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749

Tables

Table 1: Description of sampling locations including its coordinates within each river basin. Details about if there is official or non-official reports about the *Silurus* presence and results using the new molecular markers are shown *(P for positive and N for negative).*

River Basins	**Sampling Locations**	**Sample Name**	**Coordinates**	**Official Report**	**Non-Official Report**	**SilPS16S Fw/Rv**	**SilPSCO IFw/Rv**
Ebro Basin	Utebo (Zaragoza, Spain)	1.1	41.736952, -0.992233	(Doadrio 2001)	-	N	-
	Zaragoza (Zaragoza, Spain)	1.2	41.658574, -0.878066	(Doadrio 2001)	-	P	P
	Ullibarri-Gamboa (Álava, Spain)	1.3	42.938747, -2.606316	(Asensio *et al.* 1995)	-	P	P
	Nanclares de Gamboa (Álava, Spain)	1.4	42.923154, -2.576146	(Asensio *et al.* 1995)	-	N	N

River Basins	Sampling Locations	Sample Name	Coordinates	Official Report	Non-Official Report	SilPS16S Fw/Rv	SilPSCOI Fw/Rv
Tagus Basin	Villarreal de San Carlos (Cáceres, Spain)	2.1	39.83184, -6.03338	(Pérez-Bote & Roso 2009, 2011)	-	P	P
	Serradilla (Cáceres, Spain)	2.2	39.791, -6.12782	(Pérez-Bote & Roso 2009, 2011)	-	P	P
	San Martín de la Vega (Madrid, Spain)	2.3	40.2157, -3.56291	-	(Plataforma Jarama Vivo 2001)	P	P
Douro Basin	Ricobayo reservoir (Zamora, Spain)	3.1	41.53796, -5.97276	-	(PescaLeón 2010)	P	P

River Basins	Sampling Locations	Sample Name	Coordinates	Official Report	Non-Official Report	SilPS16S Fw/Rv	SilPSCO IFw/Rv
	Aldeanueva del Codonal (Segovia, Spain)	3.2	41.08061, -4.54273	-	(El Norte de Castilla 2015c)	N	N
Loire Basin	Loire river (France)	5	47.7745, 1.63367	(Krieg *et al.* 2000; Syväranta *et al.* 2010)	-	P	P
Negative Controls	San Andrés de los Tacones (Asturias, Spain)	SAT	43.501883, -5.753548	-	-	N	N
	Nora River (Asturias, Spain)	NRA	43.401321, -5.822816	-	-	N	N
	Gijón (Asturias, Spain)	4	43.544737, -5.693349	-	-	N	N

Table 2: Results of primer designs and PCR conditions to develop specific primers used in tests for early detection of *Silurus* sp. in Iberian freshwater ecosystems.

Software	**Gene**	**Primer Names**	**Sequences**	**Amplicon size (bp)**	**Annealing Temperatures**	**$MgCl_2$**
Primer-BLAST	16S	silPB16SFw	5'- ATGAATGGTGGAACGAGGGC -3	303	65ºC	2.5Mm
		silPB16SRv	5'- GCTGGTGGCCGGATCTTAG -3'			
Prise2		silPS16SFw	5'- CGTGCAGAAGCGGACATATT -3'	219	65ºC	2.5Mm
		silPS16SRv	5'- TCAGATGTTCTGTGGCTTAGAA -3'			
Primer-BLAST	COI	silPBCOIFw	5'- GCAGGAACAGGATGAACCGT -3'	239	68ºC	1.5Mm
		silPBCOIRv	5'- ATCGGCAGGGACAGGAGTAA -3'			
Prise2		silPSCOIFw	5'- TCGGAGGGTTTGGAAACTGGCTTGTG -3'	150	70ºC	1Mm

Software	**Gene**	**Primer Names**	**Sequences**	**Amplicon size (bp)**	**Annealing Temperatures**	**$MgCl_2$**
		silPSCOIRv	5'- CTGTTCCTGCGCCCGCTTCG -3			

750

Table 3: Genetic identifications using Blast in NCBI database after sequencing of the bands obtained from eDNA samples coming from in situ tests using the primers designed in this pilot study for detecting Silurus (**silPS16S**, **silPSCOI**).

DNA procedence	**Location (GPS coordinates)**	**Collector**	**Sample name**	**Gene**	**Results of assignments in GenBank database**	**GenBank number**	**% Similarity**	**% Query cover**
Tissue			2	16S	*Silurus glanis*	KR476979.1	100	97
			A	16S	*Silurus glanis*	KR476979.1	99	100
				COI	*Silurus glanis*	KR477278.1	96	99
Experimental Tanks	Beaugency (France) 47.7745, 1.63367	Quentin Mauvisseau	B	16S	*Silurus glanis*	KR476979.1	99	100
				COI	*Silurus glanis*	KR477278.1	99	99
			5	16S	*Silurus glanis*	KR476979.1	99	100
				COI	*Silurus glanis*	KR477278.1	96	98
	Zaragoza 41.6691, -0.8986	Acuario de Zaragoza	C	16S	*Silurus glanis*	KR476979.1	99	100

	Zaragoza 41.6691, -0.8986	Acuario de Zaragoza	SZ2	16S	*Silurus glanis*	KR476979.1	99	99
				COI	*Silurus glanis*	KR477278.1	96	99
eDNA	San Martín de la Vega (Madrid) 40.2157, -3.56291	Laura Miralles	2.3	16S	*Silurus glanis*	KR476979.1	99	100
	Aldeanueva del Codonal (Segovia) 41.08061, -4.54273	Laura Miralles	3.2	16S	*Silurus glanis*	KR476979.1	99	100
	Villarreal de San Carlos (Cáceres) 39.83184, -6.03338	Marina Parrondo	2.1	16S	*Silurus glanis*	KR476979.1	99	100
	Serradilla (Cáceres) 39.791, -6.12782	Marina Parrondo	2.2	16S	*Silurus glanis*	KR476979.1	97	99
	Embalse de Ricobayo (Zamora) 41.08061, -4.54273	Marina Parrondo	3.1	COI	*Silurus glanis*	KR477278.1	97	99

751 ## Figure Captions

752

753 **Figure 1**: Patterns about introduction and spreading of *S. glanis* in Iberian Peninsula
754 since 1974 until nowadays. a) Green: natural distribution of the wels catfish;
755 Red: First release in Segre River (Ebro Basin) of *S. glanis* in 1974. b) Silurus
756 presence reports in the Ebro basin from 1975 to 2000. c) Dispersal to other
757 basins: Tagus (2001), Guadalquivir (2005), Jucar (2009) and Douro (2010).
758 d) Current state (2011-2016) of *S. glanis* in the Iberian freshwater
759 ecosystems. Green dots: Official reports; Yellow triangles: Non-official
760 reports; Black borders: Sampling locations used in this work; White dots:
761 Negative controls used in this work.

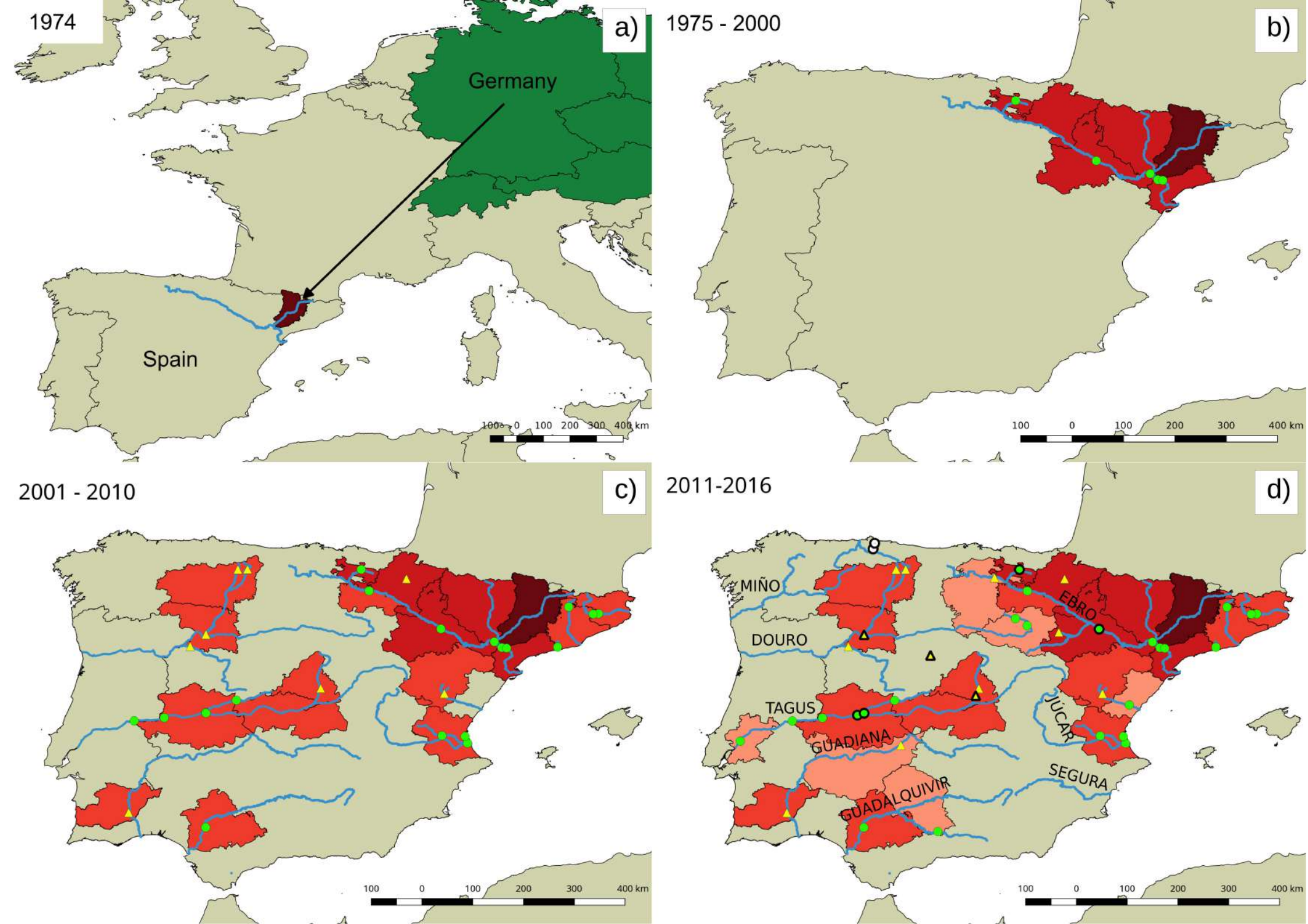
762

763 **Figure 2**: PCR results after amplifications on DNA extracts from tissues samples of *S.*
764 *glanis* and *S. aristotelis*. From 1 to 4: DNA from different individuals of *S.*
765 *aristotelis*. From 5 to 8: DNA from different individuals of *S. glanis*. In all the
766 cases there is only one specific band with the expected size for each primer
767 pair.

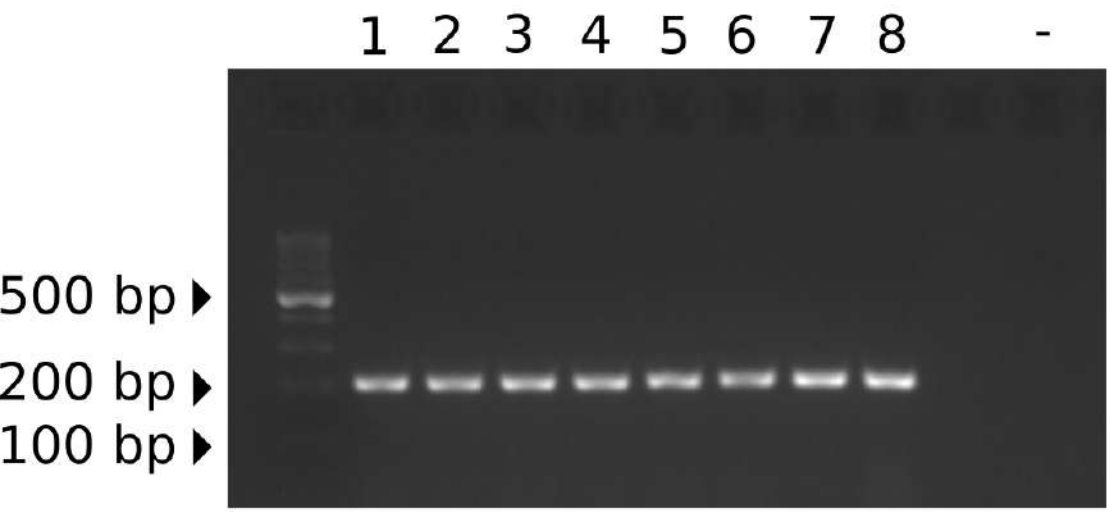
Figure 3: PCR results of the amplifications on DNA extracts from tissues samples of *S. glanis* and S. aristotelis and other eight different species that share the same habitat with *S. glanis* in Spain. Lines: 1: *Alburnus alburnus*; 2: *Scardinius erythrophthalmus*; 3: *Squalius pyrenaicus*; 4: *Leuciscus idus*; 5: *Phoxinus sp*; 6: *Pseudorasvora parva*; 7: *Carassius auratus*; 8: *Ameiurus melas*; 9-10: *S. aristotelis*; 11-12: *S. glanis*.

Figure 4: Primer pair's silPS16SFw/Rv and silPSCOIFw/Rv tests on environmental DNA samples. A: *S. glanis* (tissue); B: Artificial lab positive control; C: Water from the Aquarium of Zaragoza; -: Negative control. Samples from the Ebro Basin: 1.1: Utebo; 1.2: Zaragoza; 1.3: Ullíbarri-Gamboa; 1.4: Nanclares de Gamboa. From Tagus Basin: 2.1: Villarreal de San Carlos; 2.2: Serradilla; 2.3: San Martín de la Vega. From Douro Basin: 3.1: Ricobayo; 3.2: Aldeanueva del Codonal. Sample 4: Gijón. Sample 5: Beaugency (France).

783


1974
a)
Germany
Spain
100 0 100 200 300 400 km
1975 - 2000
b)
100 0 100 200 300 400 km
2001 - 2010
c)
100 0 100 200 300 400 km
2011-2016
d)
MIÑO
DOURO
TAGUS
GUADIANA
GUADALQUIVIR
SEGURA
JÚCAR
EBRO
100 0 100 200 300 400 km

16S gene
silPS16SFw/Rv
219 bp



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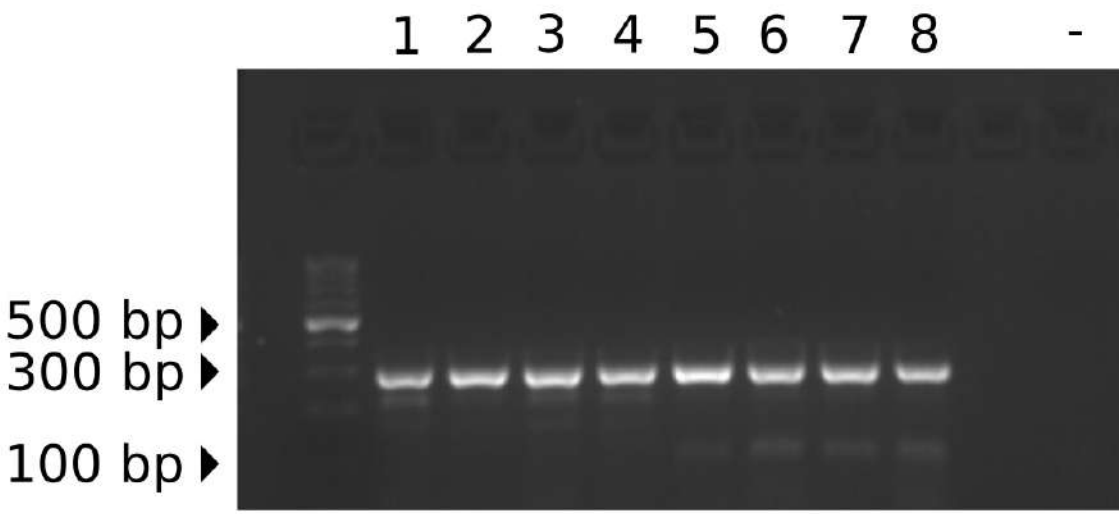
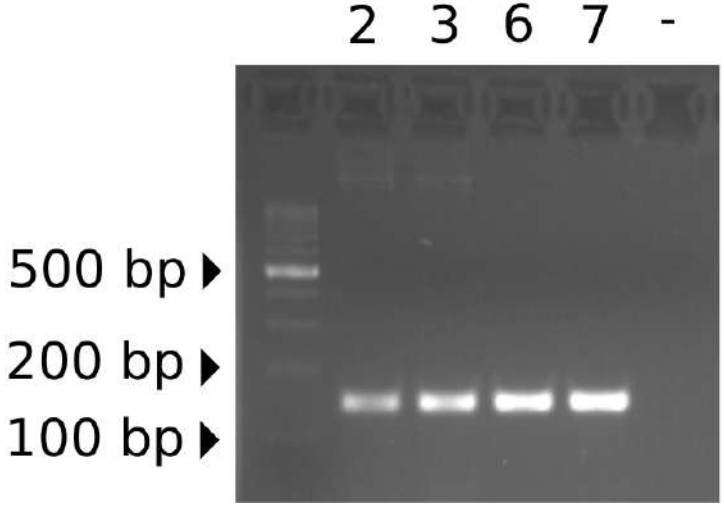
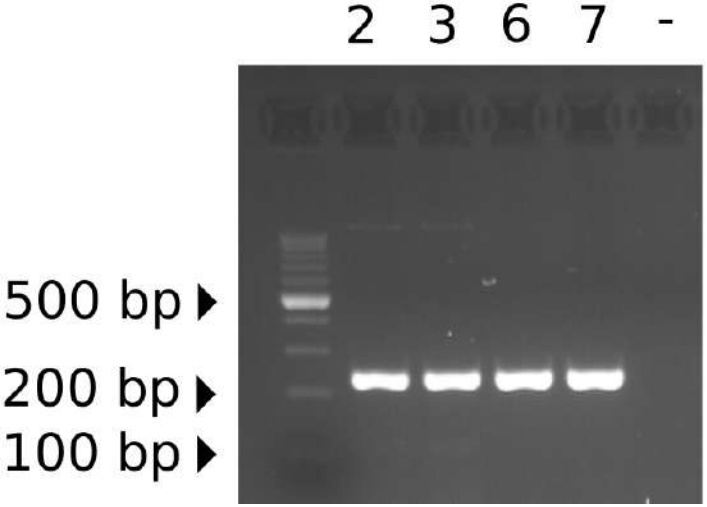
silPB16SFw/Rv
303 bp



COI gene
silPSCOIFw/Rv
150 bp



COI gene
silPBCOIFw/Rv
239 bp



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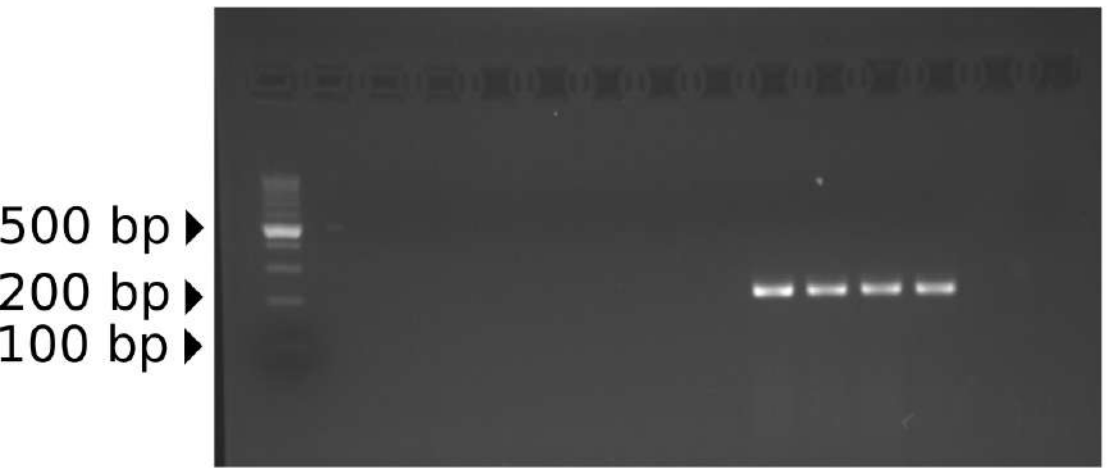
silPS16SFw/Rv
219 bp



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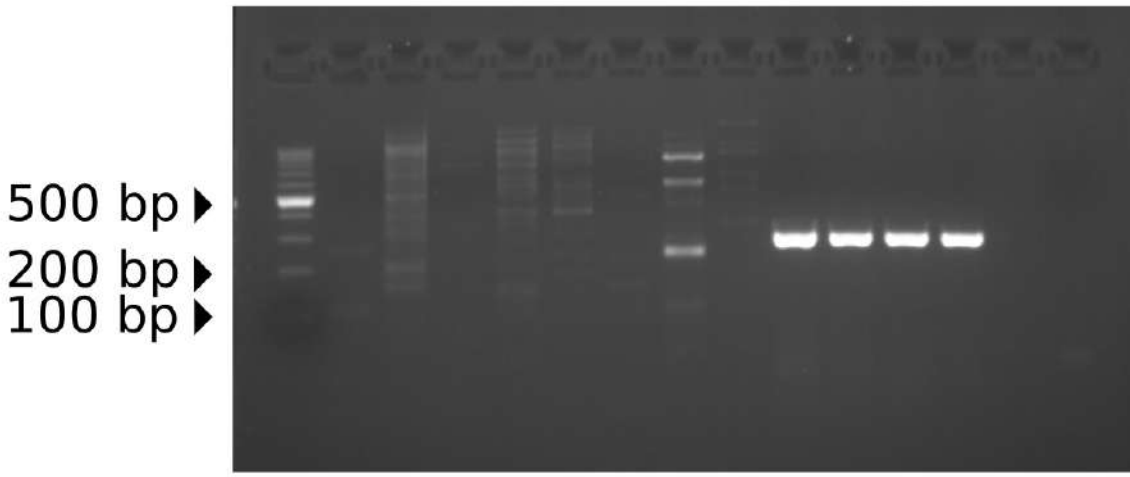
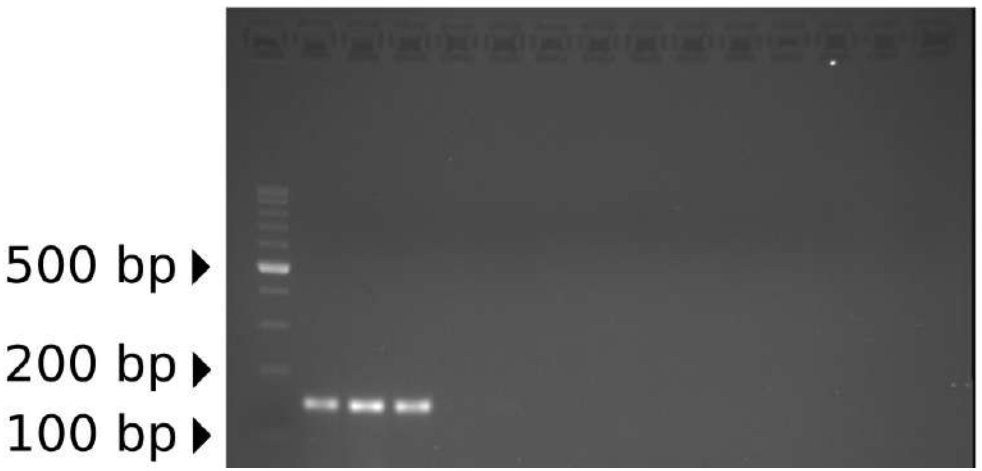
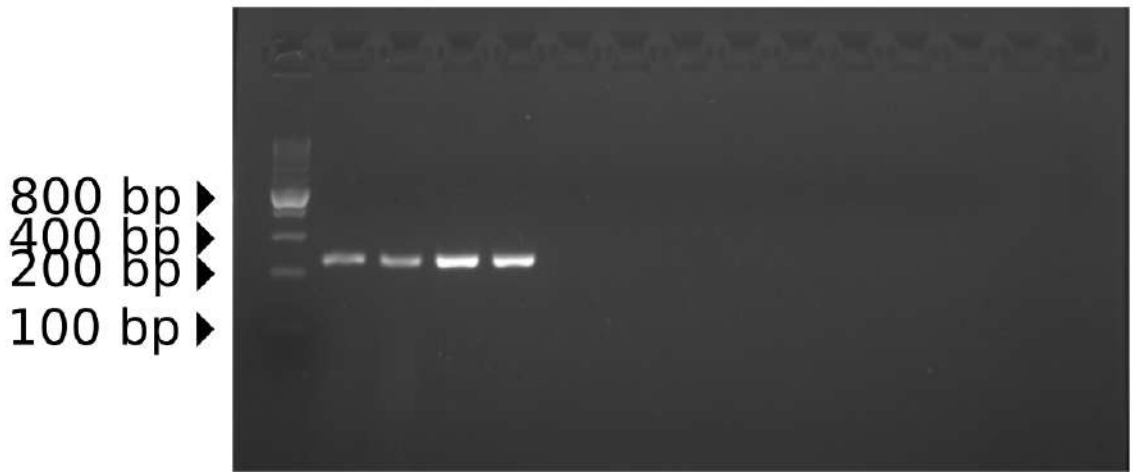
silPB16SFw/Rv
303 bp

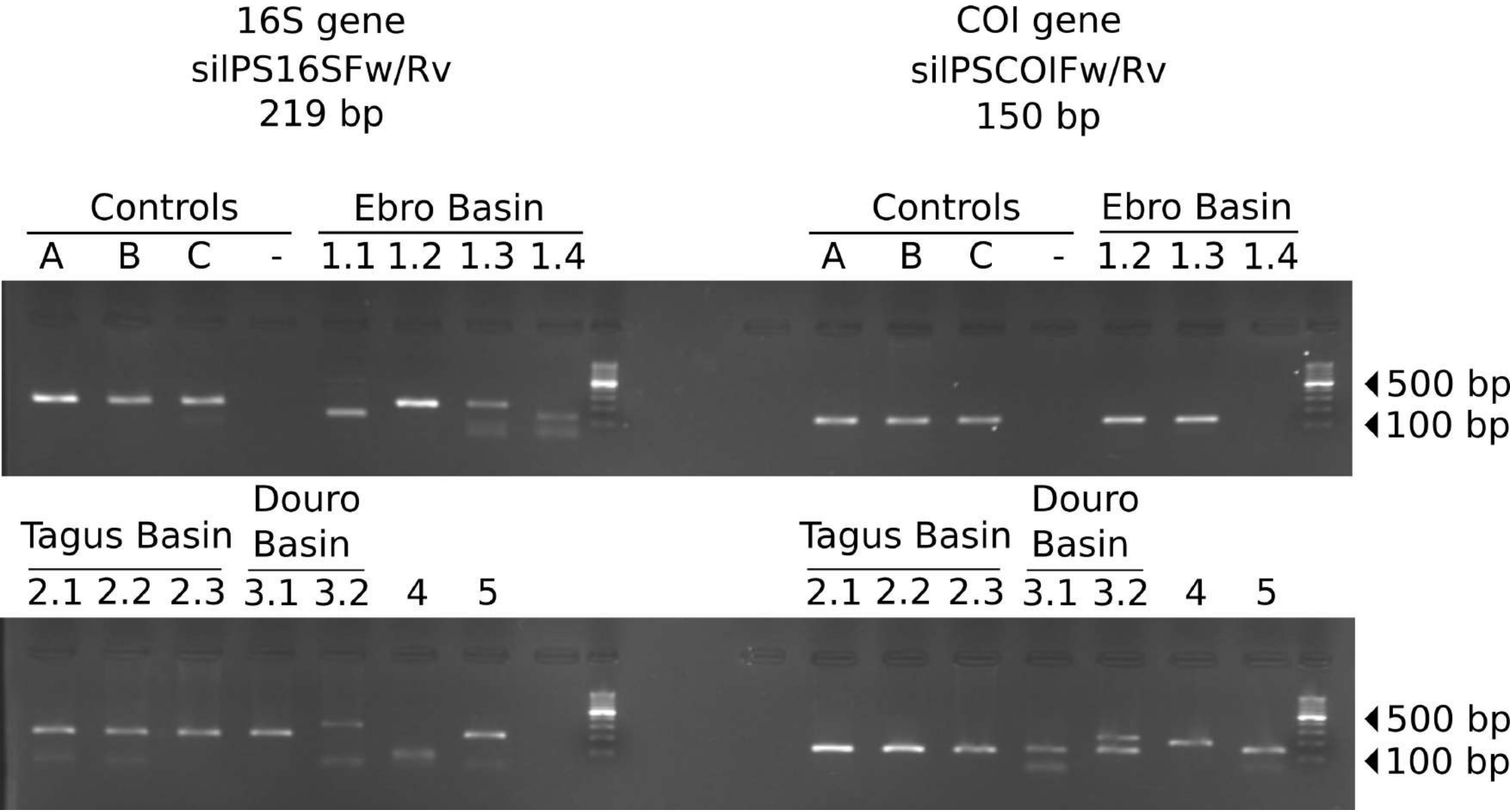


COI gene
silPSCOIFw/Rv
150 bp



COI gene
silPBCOIFw/Rv
239 bp




16S gene
silPS16SFw/Rv
219 bp
COI gene
silPSCOIFw/Rv
150 bp
Controls
Ebro Basin
A B C - 1.1 1.2 1.3 1.4
Controls
Ebro Basin
A B C - 1.2 1.3 1.4
500 bp
100 bp
Tagus Basin
Douro Basin
2.1 2.2 2.3 3.1 3.2 4 5
Tagus Basin
Douro Basin
2.1 2.2 2.3 3.1 3.2 4 5
500 bp
100 bp