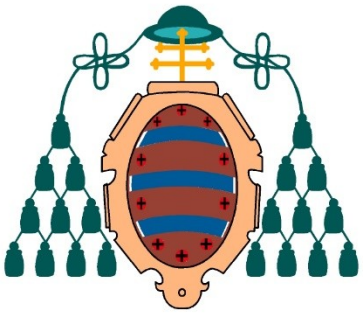

UNIVERSIDAD DE OVIEDO

MASTER OF SCIENCE IN MARINE CONSERVATION

Máster Universitario en Conservación Marina

INVASION PATTERNS OF THE MONSTER WELS CATFISH *Silurus glanis* IN SPANISH FRESHWATER ECOSYSTEMS AND DEVELOPMENT OF NEW MOLECULAR TOOLS FOR ITS EARLY DETECTION

Patrones de invasión del siluro (*Silurus glanis*) en los ecosistemas españoles de agua dulce y desarrollo de nuevas técnicas moleculares para su detección temprana

MASTER THESIS

Trabajo Fin de Máster

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Invasion patterns of the monster wels catfish *Silurus glanis* in Spanish freshwater ecosystems and development of new molecular tools for its early detection

Abstract

One of the most important threats to Spanish freshwater ecosystems are non-indigenous species (NIS). They could produce a high impact on the survival of native species as a consequence of competition for limited resources and environmental changes. The giant wels catfish *Silurus glanis*, was intentionally introduced in Spain in 1974 and, since then, it has had an alarming and hidden spread in Spanish basins due to its consideration as a fishing trophy, specially in the last fifteen years. In this work, we have updated the invasive pattern of *S. glanis* through Spanish freshwater ecosystems since the first introduction until nowadays. For that purpose, we compiled all the official and non-official information about the spread of this catfish in Spain. The current situation reflects its presence in six of the seven main river basins in the Spanish territory. Also, we have designed genus specific primers with ***Prise2*** to be used as a tool for PCRs allowing detection of this species in environmental DNA (eDNA) (a technique that allows the detection of remaining species DNA in water). They were tested on eDNA samples extracted from experimental aquariums and on real environmental samples taken from different basins in Spain (Ebro, Douro, Tagus). In all of these basins we were able to detect *S. glanis*. 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-officials reports coming from fishermen websites and newspaper reports. The eDNA method can be a helpful tool in the fight against this controversial and dangerous invasive species. It can be used for its early detection allowing fast and effective stakeholders management actions.

Keywords: Invasive species; Non indigenous species; Iberian Peninsula; environmental DNA; Management

Introduction

In Spain there are 20 hydrographic basins with different surfaces areas where more than 26 million people (55.38% of Spain population) live. The rest of the population live near the coast but also depends on the water supply of these basins (Vidal-Abarca & Suárez 2013). Virtually all activities in which humans are involved, require water for their development, not only for human and animal use but also for irrigation, power generation, recreational uses or development of products by industry, among others. All these activities are necessary to keep an adequate level of human life, but they also interfere with nature, exerting pressure on the environment. Vörösmarty et al. (2010) have argued that approximately 80% of the world's population is susceptible to high levels of threat to water security and grouped the main freshwater threatens worldwide under four themes: catchment disturbance, pollution, water resource development and biotic factors. The exotic species are among the main biotic factors affecting water security and are defined as taxa that are introduced outside their natural range, either intentionally or unintentionally by human agency (ISSG 2000). Only 1% of the total of exchanged species become invasive, being able to establish in a habitat or ecosystem and threatening native biodiversity (ISSG 2000; Mooney & Cleland 2001). Those water ecosystems that are highly altered by human activities are also the most invaded ones. Along with this low environmental diversity they present low hydrological variability, and this all favours the establishment of reproducing populations of invasive species (Ribeiro *et al.* 2008). The invasive process is developed as a multistage operation that includes the acquisition of a propagule in its native area, the transport of that propagule to the new one, and the introduction, establishment and spread of the invader in the new habitat. Alien fishes were introduced in Spain for different purposes, especially in the 20th century. Those reasons were for example: ornament, aquaculture, biological control or even by accident, but the most important pathway of fish introduction in Spain was angling (Elvira & Almodovar 2001; Maceda-Veiga *et al.* 2010). A particularly alarming case has been the introduction and spreading in Spanish freshwater ecosystems of the catfish *Silurus glanis*.

The species *S. glanis*, also known as wels catfish or sheatfish, is the largest freshwater fish in Europe and, together with *S. aristotelis*, they represent the *Silurus* genus in Europe (Triantafyllidis, Abatzopoulos & Economidis 1999; Triantafyllidis *et al.* 2002; Copp *et al.* 2009). Its natural distribution cover from the river Rhine eastwards including Asia, but it is most abundant in the Danube and Volga river basins (Triantafyllidis *et al.* 1999; Carol *et al.* 2009; Alp *et al.* 2011). The *Silurus* can reach large sizes depending on environmental conditions and the largest individuals may be above 2m long. Males are larger than females and reach sexual maturity earlier (Alp, Kara & Büyükçapar 2004; Alp *et al.* 2011; Copp *et al.* 2009; Carol *et al.* 2009). They reach

sexual maturity at 2-3 years of age and they can spend long periods of hypoxia depending on water temperature. They are one of the biggest fish predators in rivers, where they can live up to 80 years, although their life span is usually not longer than 25 years (Forgue, Burtin & Massabuau 1989; Copp *et al.* 2009; Carol *et al.* 2009; Alp *et al.* 2011). The wels catfish has been widely introduced out of its native range due to its popularity among anglers, given their large size and relatively frequent capture (Copp *et al.* 2009). In Europe, *S. glanis* has been introduced in France, Italy, the Netherlands, Belgium, Spain and the United Kingdom (Elvira & Almodóvar 2001; Alp *et al.* 2011). The species is robust enough during transport, allowing its translocation to other areas outside its native range. Once introduced, *S. glanis* seems to establish easily, being this establishment favored with warm temperatures and, because of that, it occurs less often in northern countries (Crivelli 1995; Elvira 2001; Copp *et al.* 2009) It is difficult to catch it using traditional methods as nets or electric angling (Pérez-Bote & Roso 2011). In addition, the fish is ussually discovered years after the introduction, when the population have reached high densities which difficulty the management plans or eradication attemps (Adrian-Kalchhauser & Burkhardt-Holm 2016). The potential impacts of catfish in non-native areas have been related to disease transmission (Blanc 1997; Reading *et al.* 2012), hybridization with native species (in this case limited to Greece, where cohabits with Aristotle's catfish) (Paschos *et al.* 2004), predation of native species as cyprinid fish species, mollusks, crayfish and also birds and small mammals (Adamek, Fašaić & Siddiqui 1999; Carol *et al.* 2009; Syväranta *et al.* 2010) and also with the possibility of changing the structure of the food chain in some regions. It has also been seen that in those places where catfish is present, there is a decline in the abundance of waterbirds (Copp *et al.* 2009; Carol *et al.* 2009). The catfish was originally introduced into the Iberian Peninsula in 1974 through the Segre River by Roland Lorkowsky (a German biologist) and soon after that at the Mequinenza-Ribarroja and in Flix Reservoir (Doadrio 2001; Carol 2007). After this initial spread, introductions were reported later in the Catalonia coastal basins as Sau-Susqueda Reservoirs and Llobregat River (Carol *et al.* 2003; Benejam *et al.* 2007). 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 rumoured about the spread of wels catfish in different areas of Tagus river basin (Pérez-Bote & Roso 2011) and it has been recently reported also in the Guadalquivir River (Moreno-Valcárcel, Miguel & Fernández-Delgado 2013). The spread of alien invasive fishes does not respect political boundaries and in 2015 *Silurus* reached Portugal through downstream movement along the Tagus River from populations in Spain (Gkenas *et al.* 2015) (Figure 1). Official and scientific reports seem to be out of date and not efficient at all to quickly add (and check) all the reports coming from fishermen or other citizens in web blogs, press and other media, leaving an unclear picture about this phenomenon behind. Remarkably, the Iberian Peninsula shows great levels of endemism, and native fishes have evolved without the presence of native piscivores (Crivelli 1995; Doadrio 2001; Clavero, Blanco-Garrido &

Prenda 2004; Copp *et al.* 2009). From this, the impact of catfish on Spanish freshwaters can be much greater than in the case of other European countries. This makes the need for rigorous and exhaustive species assessments urgent, and are also important for early detection of this invasive species.

The use of PCR has made a breakthrough in species identification with regard to traditional morphological identification (Taberlet *et al.* 2012). For that purpose, universal primers that amplify standardized regions allow us to know the composition of the communities that are present in an ecosystem, and this is through techniques such as (meta)barcoding (Hebert & Gregory 2005; Taberlet *et al.* 2012). However some challenges remain, such as barcoding capability to accurately identify organisms to the species level. 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 increasing accuracy and reducing the cost and time required (Ficetola *et al.* 2008). Environmental DNA (eDNA) assays have been used lately for revealing the presence of species owing to that the organism's vestigial particles remain in the environment without the need to catch those organism (Adrian-Kalchhauser & Burkhardt-Holm 2016). The development of specific primers seems to be a highly sensitive tool for detecting species in eDNA from environmental samples (Farrington *et al.* 2015). The eDNA seems to be notably appropriated for migratory species, species that are rare, invasive species or species which are hard to detect with conventional sampling tools and techniques (Bohmann *et al.* 2014; Ardura *et al.* 2015; Adrian-Kalchhauser & Burkhardt-Holm 2016). The eDNA assays requires less time, equipment, man-power, skills and financial resources than the traditional monitoring methods such as electrofishing, angling or diving. Samples can be collected by untrained people, a taxonomist is not necessary (but convenient) and the assay can be accomplished on a simple thermocycler (Bohmann *et al.* 2014; Adrian-Kalchhauser & Burkhardt-Holm 2016). Combined approaches would be useful; reports from citizens and environmental DNA tools could work together pursuing the goal of efficient detection, prevention and management policies for this monster invasive fish.

Two main aims are the leitmotivs of this study: (i) to update the current situation of the invasive wels catfish *S. glanis* in Spanish freshwater ecosystems and (ii) to design and test specific primers for *S. glanis* that allows its use as simple molecular tools to detect *Silurus* in water samples using the eDNA from rivers, lakes, artificial ponds etc. This easy and simple molecular tool would be useful for alerts (or confirmations) in early stages of the wels catfish *S. glanis* invasion processes in the Spanish freshwater ecosystems and could help stakeholders in taking effective management actions.

Material and Methods

An update of *Silurus* database for Spain

Banha, Ilhéu & Anastácio (2015) have seen that angling web forums are a good tool for checking useful information about the spread of non-native species (species abundance, places and methods of capture, photos, upcoming alien species introductions, etc.). An updated *Silurus* database was created for compiling all the relevant information about the *S. glanis* spreading pattern in Spain (available upon request, Addendum 1). For that purpose, 15 blogs, 6 webpages, 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 (http://www.magrama.gob.es/es/) about the spread/presence of *S. glanis* in Spain until June 2016 were consulted, scrutinized and summarized. A graphic interface (a *Silurus* spreading map) representing all the information included in this database was designed using QGIS 2.14 Essen (http://www.qgis.org/es/site/).

Primer Design

Cytochrome oxidase subunit I (COI) and 16S sequences from public databases as Genbank and BOLD (www.boldsystem.com) 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 software: ***Prise2*** (PCR Primer Design Software) (Huang *et al*. 2014) and ***Primer-BLAST*** (Ye *et al*. 2012). The following parameters were selected in both software programs: 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 70°C with a maximum difference of 2°C between forward and reverse primers and finally a percentage of GC between 25 and 75%. For the case of primer design with ***Prise2***, different settings for the 3' end were tested and those which present 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-BLAST*** primers design, specific primers that have at least two mismatches within five bases from the 3' end of the primer were chosen.

Tissue samples, DNA Extractions and PCR amplification

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 -20°C. All the individuals were barcoded using the Cytochrome oxidase subunit I (COI) gene (Ward *et al*. 2005). Genetic identifications/assignments were done using the BOLD database with species identifications over 99% of identity confirming that the tissue samples

were from six *S. aristotelis* and from eight individuals of *S. glanis*.

The specific primers developed in the previous step were used to obtain specific amplicons in tissue samples through PCR procedures in a 2720 Thermal Cycler, Applied Biosystems. For a final volume of 20µL, Green Go Taq Flexi Buffer (1X) PROMEGA®, $MgCl_2$ (from 1mM to 2.5mM 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 95°C during 5 minutes, followed by 35 cycles with a denaturation step at 95°C during 30 seconds, 30 seconds of annealing between 65°C to 70°C (depending of each primer pair) and an extension step at 72°C during 30 seconds. Finally the PCR cycling end with a final elongation at 72°C for 7 minutes. PCR products were checked on a 2% agarose gel stained with SimplySafe™.

Primer Specificity Test on other fish tissues

Fish tissue samples from eight other fishes that usually share their habitat with *Silurus sp.* in Spain (*Alburnus alburnus; Scardinius erythrophthalmus; Squalius pyrenaicus; Leuciscus idus; Phoxinus phoxinus; Pseudorasbora parva; Carassius auratus; Ameiurus melas*) (Elvira & Almodóvar 2001), were used for "on location" 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.

DNA extraction and PCR amplification procedures for water samples

In order to create our own artificial lab positive control of *S. glanis* eDNA in water, an already dead and frozen *S. glanis* fish (size: 49cm; weight 654g) was provided by a fisherman from Loire (Tours, France). After COI barcoding and genetic identification, portions of muscle from this fish (1g, 2,g, 5g, 10g and finally 20g of *S. glanis* tissue) were put in 5 different containers with 500 ml of distilled water each one for 7 days. Freshwater samples (1.5 liter) were also obtained from areas with or without reported presence of *S. glanis* (used here as positive and negative controls for the newly developed molecular tools, respectively). Samples from the Ebro (Spain) and Tagus rivers (Spain), together with the Ullibarri-Gamboa reservoir (in Vitoria, Basque Country, Spain) and also one sample from the river Loire (France) were tested and used as positive controls since all of them have been officially cited as location with *S. glanis* presence (Table 1). Freshwater samples from the Nora River and from San Andrés de los Tacones reservoir (Asturias, Spain) and also one sea water sample from the Port of Gijón where used as negative controls (there are not 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 Martín

de la Vega lagoon (Madrid, Spain) and Aldeanueva del Codonal lagoon (Segovia, Spain) (Table 1).

Water samples were taken in sterile plastic bottles just below the surface. The coordinates of each sampling point were recorded and the bottles were properly labelled and stored in cold ice for transportation to the laboratory, where they were immediately frozen until the filtering process. The water samples 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. The DNA was isolated from water samples using the PowerWater® DNA Isolation Kit (MO-BIO), following the manufacturer protocol. 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 et al. (2005) recommendations when working with eDNA from natural freshwater samples. The PCR program were just slightly modified increasing the number of the three step cycling (55 times) (Thomsen *et al*. 2012; Takahara, Minamoto & Doi 2015). 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, as in Ardura et al. (2015).

Results

The current situation of the catffish *S. glanis* invasion process in Spain

The inland territory of Spain is politically divided in fifteen communities from which all, except the Principality of Asturias, are crossed by seven main rivers/watersheds (Ebro, Douro, Tagus, Guadiana, Guadalquivir, Jucar and Segura). The Figure 1 summarizes the evolution of invasive process of the species *S. glanis* in Spain since the first release of 32 young individuals in 1974 in the Segre River (Figure 1a) (Carol 2007) until nowadays (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 one community was affected by the *Silurus* invasion (Catalonia), but at the end of the year 2000, the *S. glanis* presence was already officially reported at 4 out of the 14 Spanish communities (≈ 29%): Catalonia, Aragon (Doadrio 2001; Elvira & Almodovar 2001), Navarre (Navarra 1997) and Basque Country (Asensio, Pinedo & Markina 1995). The possibility of its presence in La Rioja was also commented in an official report (Zaldívar 1994) which could increase the percentage of affected communities to 36% of the inland Spanish territory. Before the year 2000 non-official reports about *Silurus* in Spain were really scarce (Figure 1b).

A dramatically expansion of the *Silurus* invasion process is evident for the last 16 years (Figure 1c and

1d). The species was officially recorded in nine Spanish communities (64%) from year 2000 to 2010. There were several new presence reports out of the Ebro basin in Catalonia (Carol *et al.* 2003; Benejam *et al.* 2007; Aparicio & Julià 2009). It was reported in Castile-La Mancha (Nicola *et al.* 2009), Extremadura (Doadrio 2001; Pérez-Bote & Roso 2009), Andalusia (Alegre & Ceballos 2006) and Valencia (Comunitat Valenciana 2009, 2010). Moreover, non-official reports increase this occupation percentages to eleven of the Spanish communities (78%) adding presence registers in Madrid (Plataforma Jarama Vivo 2001) and Castile and Leon (PescaLeón 2010). At the end of 2010, four of the seven main river Spanish basins were officially reported as invaded by *S. glanis* (Ebro, Tagus, Guadalquivir and Jucar) and in a non-officially way it was also reported by fishermen in four different reservoirs of the Douro watershed (Figure 1c).

The Figure 1d shows a summary of the *S. glanis* presence reports done between 2011 and 2016. There are not reports about *S. glanis* in Murcia (the Segura watershed), Cantabria (origin of the Ebro river) or in Galicia (influenced by the Duero river) yet. However, *S. glanis* is still in expansion with new official reports in many other regions within Castile and Leon, specifically in the area surrounding the city of Soria (Diario de Soria 2014; Junta de Castilla y León 2015; Tardajos de Duero 2015; El Norte de Castilla 2015b). There was also a second official report in the Guadalquivir River (Moreno-Valcárcel *et al.* 2013) and its presence in the Sitjar reservoir in Castellón (Valencia) was also officially reported (Levante-EMV 2012). The first 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 (Ieltxu Vega 2011; Hay Pesca! 2012), Extremadura (Navalmoral Digital 2015) and Madrid (Hay Pesca! 2014; Ediciones El País 2016) (Figure 1d). The current picture about this spectacular biological invasion seems to be that nowadays six out of the seven main river basins in Spain (86%) have been invaded by *S. glanis*. The unique exception is the Segura watershed where none official or non-official report has alerted until now about *S. glanis* presence.

Developing specific primers for early detection of *S. glanis* in Spain

Four genetic markers (different primer pairs) were designed in this work for specific detection of *S. glanis* in the eDNA. They were the result of using two different primers designing softwares (***Prise2*** and ***Primer-BLAST***) and two genes: the mitochondrial cytochrome oxidase I (COI) and the ribosomal gene 16S. The design of specific primers can resulted in different levels of specificity (species, genus, family, order) in the resulting primer pairs; this depends on the levels of genetic variation found in the genes being under consideration. 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. Both software assays were not 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*, *Silurus biwaensis*, *Silurus lanzhouensis* and *Silurus meridionalis*. This primer pair (**silPB16s**) was then 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 no native from Spain.

The primer design results from ***Primer-BLAST*** when working with the COI gene (Table 2) were similar to these previously achieved with the 16S gene since they were just genus specific (**silPBCOI**). They potentially will 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).

Primer specificities were also assayed with DNA extracted from tissues of *S. glanis* and *S. aristotelis* (Figure 2). In vitro PCR assays demonstrated that the four primer pairs produced the expected PCR amplicons (expected sizes) and besides this, they did not show any unspecific band pattern (Figure 2). Additional analysis were done in this work to demonstrate the absence of PCR artifacts 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.

The eDNA was successfully extracted from water samples coming from two tanks containing *Silurus* (positive eDNA controls: the Zaragoza aquarium and the Lab positive control done using tissues from a *Silurus* fish) and also from three Spanish basins (Duero (2 samples), Ebro (4 samples) and Tagus (3 samples)) and one French river showing *Silurus* presence (Loire River). Primers pairs developed in this work (**silPS16s**, **silPSCOI**) were assayed with these samples and revealed successful PCR results (Figure 4). The *S. glanis* tissue (A), the artificial lab eDNA positive control (B) and the aquarium of Zaragoza eDNA sample (C) yielded a unique band at the expected sizes (COI gene: 150bp and 16S gene: 219bp) with both primer pairs (Figure 4).

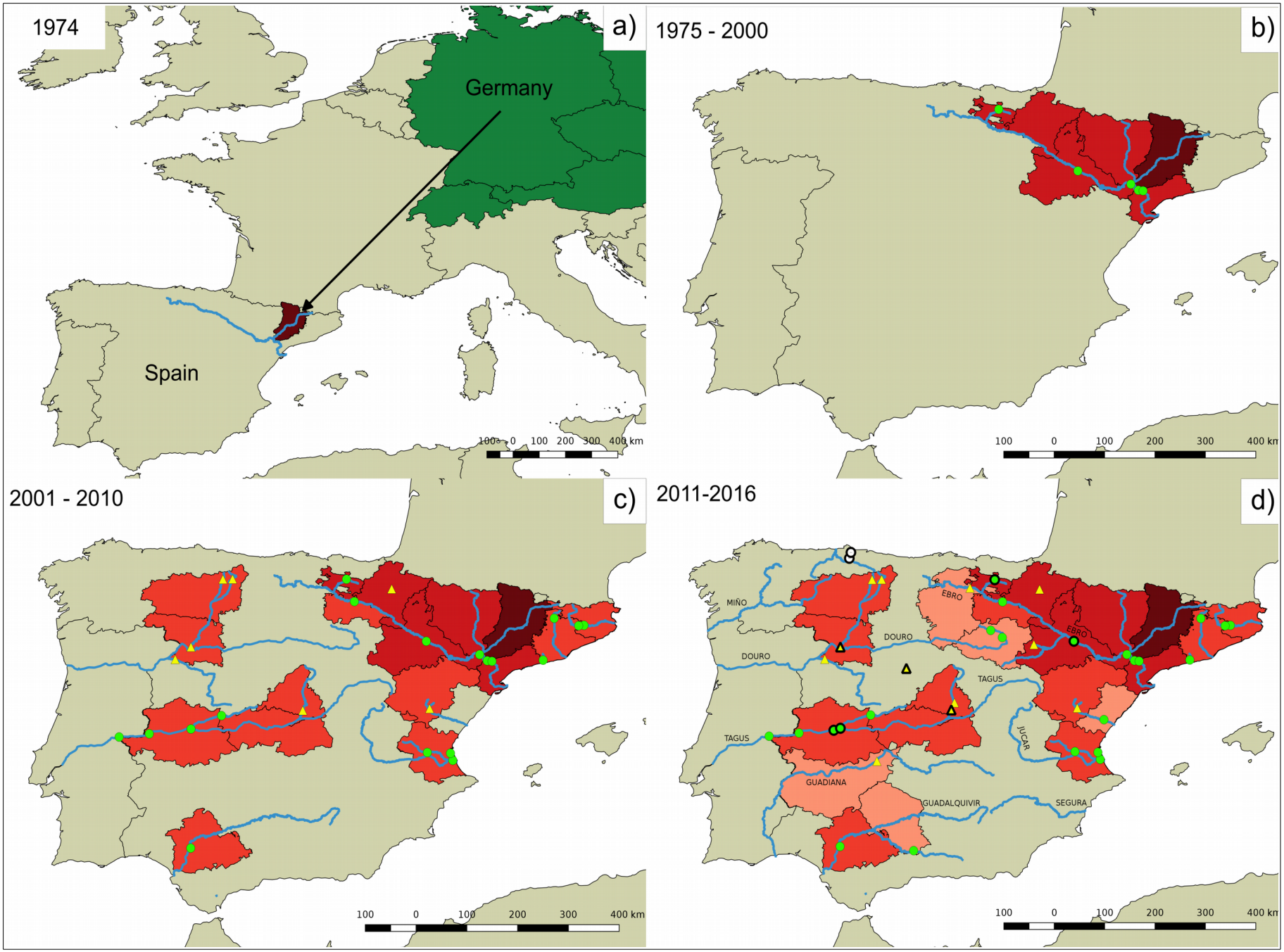

Figure 1: Patterns about introduction and spreading of *S. glanis* in Spain since 1974 until nowadays. **a)** Green: natural distribution of the wels catfish; Red: First release in Segre River (Ebro Basin) of *S. glanis* in 1974. **b)** Silurus presence reports in the Ebro basin from 1975 to 2000. **c)**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

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	Coordinates	Official Report	Non-Official Report	SilPS16SFw/Rv	SilPSCOIFw/Rv
Ebro Basin	Utebo (Zaragoza, Spain)	41.736952, -0.992233	(Doadrio 2001)	-	N	-
	Zaragoza (Zaragoza, Spain)	41.658574, -0.878066	(Doadrio 2001)	-	P	P
	Ullibarri-Gamboa (Álava, Spain)	42.938747, -2.606316	(Asensio *et al*. 1995)	-	P	P
	Nanclares de Gamboa (Álava, Spain)	42.923154, -2.576146	(Asensio *et al*. 1995)	-	N	N
Tagus Basin	Villarreal de San Carlos (Cáceres, Spain)	39.83184, -6.03338	(Pérez-Bote & Roso 2009, 2011)	-	P	P
	Serradilla (Cáceres, Spain)	39.791, -6.12782	(Pérez-Bote & Roso 2009, 2011)	-	P	P
	San Martín de la Vega (Madrid, Spain)	40.2157, -3.56291	-	(Plataforma Jarama Vivo 2001)	P	P
Douro Basin	Ricobayo reservoir (Zamora, Spain)	41.53796, -5.97276	-	(PescaLeón 2010)	P	P
	Aldeanueva del Codonal (Segovia, Spain)	41.08061, -4.54273	-	(El Norte de Castilla 2015c)	N	N
Loire Basin	Loire river (France)	47.7745, 1.63367	(Krieg *et al*. 2000; Syväranta *et al*. 2010)	-	P	P
Negative Controls	San Andrés de los Tacones	43.501883, -5.753548	-	-	N	N
	Nora River	43.401321, -5.822816	-	-	N	N
	Gijón	43.544737, -5.693349	-	-	N	N

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

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

Three basins officially reported with *Silurus* fish presence were 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, Cáceres), 2.2 (Serradilla, Cáceres), 2.3 (San Martín de la Vega, Madrid)) all yielded positive results with both markers that were similar in shape and sizes 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).

The global level of correspondence among the expected (officially or non-officially locations reported with *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 in a 70% (**silPS16s**: 7 out of 10 assayed eDNA samples) and 77% (**silPSCOI**: 7 out of nine eDNA assayed samples) (Table 1). Official *Silurus* reports were confirmed with both markers in 5 out of 6 cases (83%) and in two out of three (66%) non-official reports coming from fishermen websites and newspaper reports (Table 1).

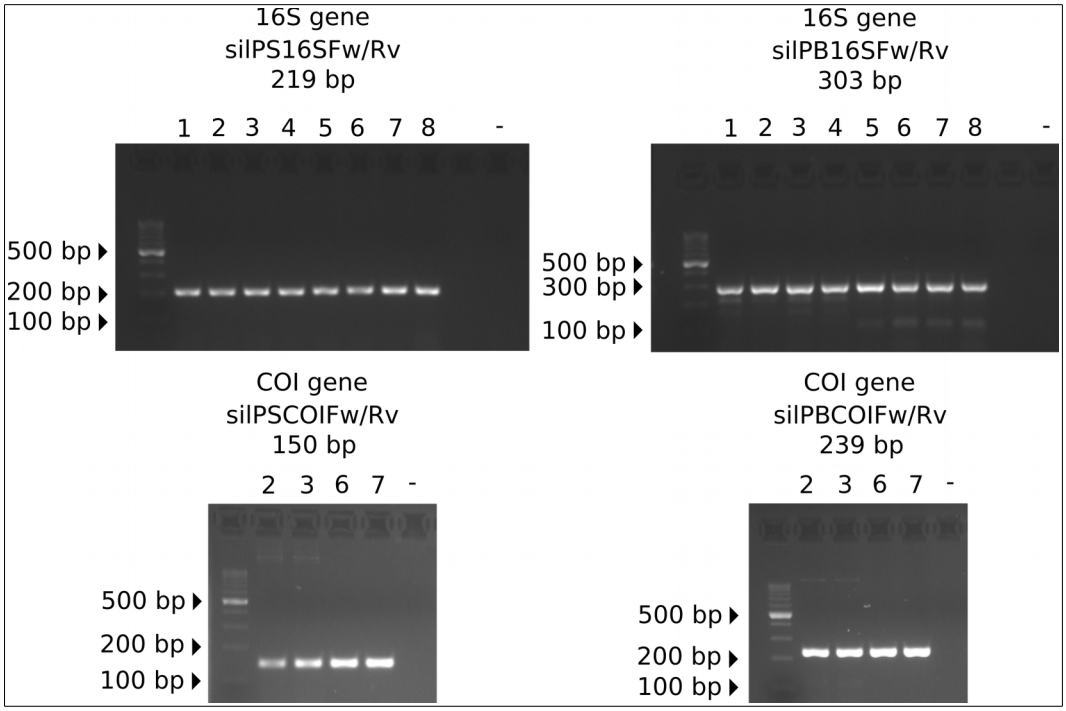

Figure 2: PCR results after amplifications on DNA extracts from tissues samples of *S. glanis* and *S. aristotelis*. From **1 to 4**: DNA from different individuals of S. aristotelis. From **5 to 8**: DNA from different individuals of *S. glanis*. In all the cases there is only one specific band with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

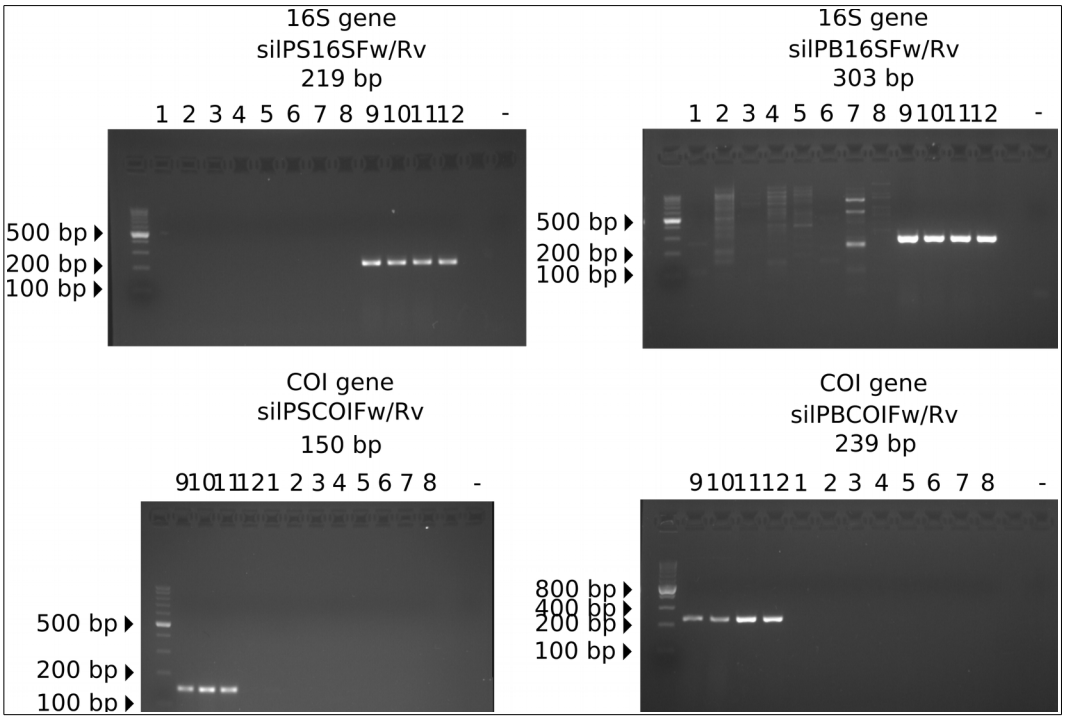

Figure 3: PCR results of the amplifications on DNA extracts from tissues samples of *S. glanis* and *S. aristotelis* and on the eight different species that share the same habitat with *S. glanis* in Spain. **Lines**: **1**: *Alburnus alburnus*; **2**: *Scardinius erythrophthalmus*; **3**: *Squalius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

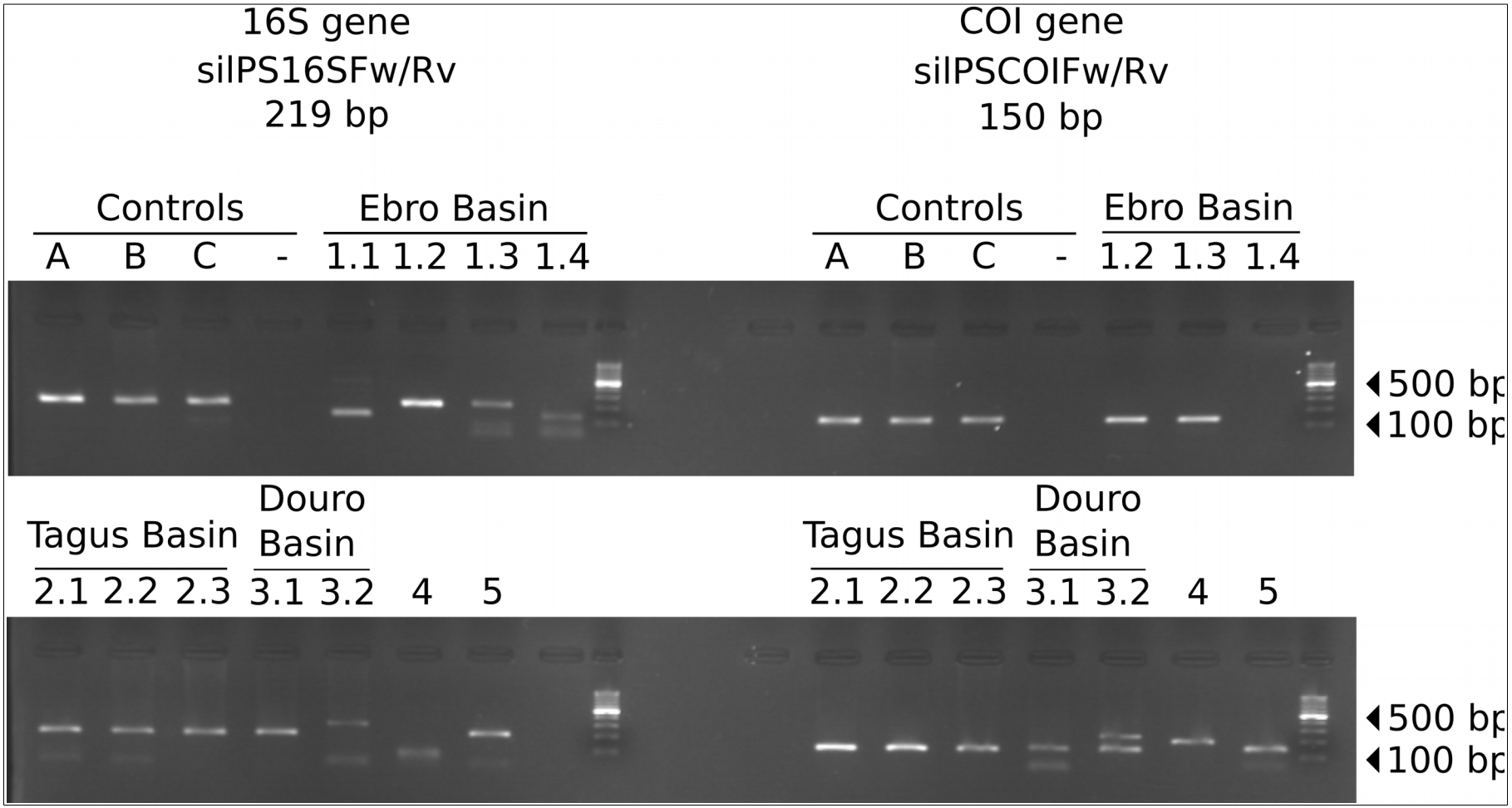

Figure 4: Primer pair's **silPS16S** and **silPSCOI** tests on environmental DNA samples. **A**: *S. glanis* (tissue); **B**: Artificial lab positive control; **C**: Water from the Aquarium of Zaragoza; **-**: Negative control. Samples from the Ebro Basin: **1.1**:Utebo; **1.2**: Zaragoza; **1.3**: Ullíbarri-Gamboa; **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).

Discussion

The distribution/presence of *S. glanis* throughout the rivers of the Iberian Peninsula is just partially reported nowadays. The Administration reports are not usually updated. This is, probably, because the available techniques do not allow detection of an invasive species until the population is established (years after the first introduction event). In this Master Thesis, the first molecular markers for the detection of *S. glanis* in eDNA coming from freshwater ecosystems from the Iberian Peninsula, independently of the stage of the introduction, has been developed. This kind of technique is cheaper than the traditional monitoring with nets or electrofishing, and thus can be a useful tool since *S. glanis* is even difficult to catch with traditional fishing techniques (Pérez-Bote & Roso 2011).

Four different pairs of molecular markers were designed in this work using two different primer design software: ***Prise2*** (Huang *et al.* 2014) and ***Primer-BLAST*** (Ye *et al.* 2012). None of these software gave species-specific molecular markers as a result because silurids are close related species, sharing high homologies for the two genes used here. This has happened before in several studies attempting to develop species specific molecular markers (i.e.: Japanese salamanders (Fukumoto, Ushimaru & Minamoto 2015)). The new molecular markers developed in this work are genus-specific markers that will allow the detection of silurids in Spanish freshwater ecosystems. Their lack of specificity for the *S. glanis* species will not be, actually, a big issue since in Spain *Silurus* sp. is not a native genus. Therefore the new molecular markers will be able to detect not only *S. glanis* as an invasive species but also other potential invasive catfishes.

The results of all the quality tests done in the course of this work (in silico, tissues and eDNA) revealed ***Prise2*** as a very efficient tool for designing specific primers to be used with eDNA allowing very specific differentiation in the selection of target and non-target species (Huang *et al.* 2014) and better experimental results. The ***Prise2*** software allows a customizable, detailed an very sophisticated selection at the 3' end of amplicons for both, target and non-target species, while ***Primer-BLAST*** only allows to select the total number of mismatches to unintended targets with many of them being present in the 3' end what can produce later unspecific band patterns in PCRs. Primer design improvements, when working with ***Primer-BLAST,*** were attempted in this work choosing more specific settings for the 3' end but it was not possible to obtain species specific results or better primer combinations.

Despite high sensitivities, the eDNA assays are susceptible to false positives and false negatives. False positives (error type I) can be explained as eDNA detections when the species of interest is not present meanwhile false negatives (error type II) appear when eDNA is not detected being the target species present in the sample (Ficetola *et al.* 2015). The Figure 4 shows that samples coming from the Ebro basin (the starting

initial introduction location for *Silurus*) were recorded as a positive for *Silurus* using both molecular markers but the exception of the sample 1.4 (Nanclares de Gamboa, Vitoria, Ebro basin). Is that a false negative? Samples were collected in different points of the same reservoir where the *Silurus* was reported since 1993 (Asensio *et al.* 1995). False negatives can be caused in some environmental samples by leaf litter, current flow or sampling depth (Darling & Mahon 2011; Bohmann *et al.* 2014; 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). It seems that *Silurus* remain near the littoral zone covered by a very dense vegetation and spend all the winter in the lower third of water column or on soft mud (Carol, Zamora & García-Berthou 2007; Copp *et al.* 2009). In this study water samples were taken during early spring in shallow water layers and next to the shore because reservoirs are not usually accessible in all their extension. Obviously, the fish are not homogeneously distributed in a reservoir which implies fish densities will be heterogeneous compromising eDNA detection accuracy. Despite this, different sampling points from the same reservoir (as it has been done in this work) will help to avoid false negatives. More studies correlating fish densities (using subaquatic cameras or echo techniques) and eDNA specific detections will help in the future to know in depth lower limits/cut-offs for eDNA detection procedures. Accurate species determinations in official and non-official reports are also needed when considering false negatives. In the sample 3.2 (Aldeanueva del Codonal, Douro basin) were reported “millions of catfishes” but later those fishes resulted to be *Ameiurus melas* (Order Siluriformes) which, to an untrained eye, it can be easily confused with *S. glanis (El Norte de Castilla 2015c a)*. The Figure 3 shows that *A. melas* was one of the species that were used to test the primers without apparent cross amplification during the development of the conditions for primers.

False positives are usually a much more serious concern in detection studies. Alarming attitudes after detections could imply the use of economic resources and the establishment of measures to fight against, or control, a biological invasion. False positives in environmental samples are difficult to scrutinize in this work since only official and non-official reports are used as *Silurus* presence evidences. However, the artificial positive samples created here worked well and gave no doubts about incurring in false detections. Non-expected size band (smaller or higher than the predicted ones) were also found in this work. This can be a results of increasing the number of PCR cycles although these bands have been also associate to asymmetric PCRs outcomes (Poddar 2000), in which only one of the two primers (forward and reverse) recognizes the eDNA strain because it is partially degraded (Dejean *et al.* 2011) and results in a small fragment of eDNA that acts as a template for the newly created strand. Upcoming experiments should, when possible, use sequencing of these no expected PCR products and compare the results with a public database such as BOLD or GenBank to assess

their nature. Finally, it will be also necessary to assess if it is possible to quantify specific eDNAs using qPCR and correlate these quantities with fish densities. It has been recently highlighted significant correlations between the amount of species eDNA detected and the biomass of the target species which is even a relevant finding and a promising tool for fisheries management (Eichmiller, Miller & Sorensen 2016).

The combination of different characteristics along the invasive process determine the success of non-native fishes in each invasive stage. Because of its atributes as high growth rate; robutness during transport; longevity; adaptable feeding habits; high fertility and parental care; high movility during juvenile stages; extremely tolerant with water quality and temperature, *S. glanis* is a really huge threat to introduced habitats (Slavík *et al.* 2007; Carol *et al.* 2007, 2009; Ribeiro *et al.* 2008; Copp *et al.* 2009; Alp *et al.* 2011). The presence of *S. glanis* in Spain have had a condierable 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; in one hand by anglers and in 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 being the Tagus-Segura the most important of all of them (Vidal-Abarca & Suárez 2013). In the Iberian Peninsula this massive damming of rivers has led to the introduction of game fishes for promoting recreational fisheries into reservoirs which do not allow the movement of individuals, changes the flow and alter the environment, resulting in a high impact on the freshwater biodiversity (Ribeiro *et al.* 2008; Vörösmarty *et al.* 2010). Preserving measures to protect the habitats usually are taken after reports about these affectation to the habitats. However, inefficient surveillance and slow alarm and control measures by the authorities are a fact in Spain. The Government of Andalusia, even when reported the capture of the two first catfishes in the port of Seville in 2005, did not officially report catfish presence but in the Guadalquivir river until 2013 (Alegre & Ceballos 2006; Moreno-Valcárcel *et al.* 2013). New reports are continuously been appearing in non-official channels and there is the need of actions to confirm/discard the presence of *Silurus* (i.e:Almendra, Ricobayo, Porma and Riaño reservoirs (PescaLeón 2010)). If true, it would be a much greater expansion for *Silurus* in Spain than previously thought. The sample rom the Ricobayo reservoir (Douro basin) used in this work (sample 3.1, Figure 4) showed *Silurus* presence with the two new molecular markers developed here. Adding this result to the official report of the presence of *Silurus* in the Douro river in the surrondings of Soria (Diario de Soria 2014; Junta de Castilla y León 2015; El Norte de Castilla 2015b; Heraldo de Soria 2015), makes urgent management measures taken by Spanish and Portuguese authorities to avoid the spread of *S. glanis* along the entire river. The presence of *Silurus* in the Guadiana Valley Natural Park, in Portugal has been already reported (Greenpeace 2006), therefore it would not be unlikely that the species had expanded along all the river and even the presence of catfish in the Guadiana watershed cannot be discarded at all. 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 strictly recommended its use, together with molecular markers such as those developed here as part of informative phases for more complete management plans dealing with this dangerous invasive species.

One of the main findings from this work is little social perception of the damage that can cause invasive species to the environment. In the course of obtaining tissue samples of *S. glanis* for testing our molecular tools, we asked for the help of at least six sport fishing associations from Tagus, Ebro, Jucar and Guadalquivir basins. We were not able to obtain any tissue samples. Fisherman argued they practice catch and release of the caught fish (despite being illegal in the case of invasive species). We also found several messages in various fish forums where fishermen were completely prided themselves on not comply with the laws established by capturing invasive species, thus favoring its expansion (Webcarp 2007). All of this support the idea of anglers as main cause of exotic species introductions in Spanis River ecoysistems. Recently, the Spanish laws about invasive species (the official Catalogue of Alien Invasive Species) has undergone some changes adding new species. This has provoked great conflicts between fishermen, scientists and institutions (including fishermen demostrations in front of government offices). When one of these species are listed in this catalogue this implies prohibitions and regulation of all the activities related with those species. Fishermen and other stakeholder think when one species support commercial activities (more in an economic crisis scenario) this help people and must be regulated just in a flexible way. People is first. They argued social impact is a priority and associated businesses related with some exotic species can move significant amounts of money and even promote employment options in depressed areas (Diario ABC 2016b a; El Mundo 2016). Nevertheless, it is more than proven that invasive species cause so much extensive damage in the new habitats (Hermoso *et al*. 2011).

Definitely, humans are not along in earth and depend (a lot) on healthy environments for surviving. Prevention, detection, correct managements actions and a considerable change in minds about the relevant issues such as how to deal with invasive species is of great importance and will define how the future of Spanish freshwater ecosystems, and obviously, of humans will be.

Conclusions

1. The species *Silurus glanis*, introduced in the Ebro basin in 1974, is currently present in six out of the seven main river watersheds of Spain (86%) (11 Autonomous Communities). An impressive and accelerated process of biological invasion has occurred since year 2000, probably as a consequence of fishermen activities and water transfers done by regional and national goverments.
2. Official reactions to the apparition of invasive species such as *S. glanis* in Spanish freshwater

ecosystems are really slow. Other sources of information about detection events (i.e: fishermen blogs, newspapers, etc), even when incomplete and not always accurate, seems be useful and must be incorporated to a surveillance system about *Silurus* biological invasion in Spain.

3. Using two different primer design softwares (***Prise2*** and ***Primer-BLAST***) we were able to develop genus-specific markers for the PCR detection of *Silurus* DNA in tissues and in eDNA coming from water samples. The software ***Prise2*** gave much better results in comparison with ***Primer-BLAST*** since primer pairs found with ***Prise2*** (**silPS16s**, **silPSCOI**) showed much more specificity and are recommended for *Silurus* detection attemps.

4. The new genetic tools developed (**silPS16s**, **silPSCOI**) are useful for the detection of *Silurus* sp. eDNA in water samples. A total of five out of six official *Silurus* reports (83%) found for different Spanish River basins were confirmed in this work by molecular test. Moreover, two out of three non-officials reports coming from fishermen websites and newspaper reports were also confirmed. The use of these new molecular marker allows the detection of *Silurus* sp. in any ontological stage which indeed suppose a decrease in costs associated to detection programs and means a considerable improvement of the management and control strategies for this species.

5. Preventing future *Silurus* introductions in all the Iberian Peninsula is necessary but will not be possible without rasing social and scientific awareness about the real threats of invasive species to freshwater ecosystems.

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Appendix

Addendum 1: Silurus glanis reports in Spanish freshwater ecosystems since the first introduction in 1974 until nowadays. In green appear those official reports while in yellow appear those non-official ones.

Report Year	Water Source	River Basin	Autonomous Community	References
1974	Segre River	Ebro	Aragon	(Carol 2007)
1974	Mequinenza-Ribarroja	Ebro	Aragon	(Doadrio 2001)
1993	Ullíbarri-Gamboa	Ebro	Basque Country – Castile and Leon	(Asensio *et al.* 1995)
1997	-	-	Navarre	(Navarra 1997)
2001	Cedillo	Tagus	Extremadura	(Doadrio 2001)
2001	San Martin de la Vega	Tagus	Madrid	(Plataforma Jarama Vivo 2001)
2003	Sau-Susqueda	Ter	Catalonia	(Carol *et al.* 2003)
2004	Valbona	Jucar	Aragon	(El Periódico de Aragón 2004)
2005	Guadalquivir	Guadalquivir	Andalusía	(Alegre & Ceballos 2006)
2006	La Baells	Llobregat	Catalonia	(Benejam *et al.* 2007)
2007	Rosarito	Tagus	Castile - La Mancha	(Nicola *et al.* 2009)
2007	Foix	Interior Basins of Catalonia	Catalonia	(Aparicio & Julià 2009)
2008	Arroyo de la Vid	Tagus	Extremadura	(Pérez-Bote & Roso 2009)
2009	Forata	Júcar	Valencia	(Comunitat Valenciana 2009, 2010; Levante-EMV 2009bdc a)
2010	Almendra	Douro	Castile and Leon	(PescaLeón 2010)
2010	Ricobayo	Douro	Castile and Leon	(PescaLeón 2010)
2010	Porma	Douro	Castile and Leon	(PescaLeón 2010)
2010	Riaño	Douro	Castile and Leon	(PescaLeón 2010)
2011	Iznájar	Guadalquivir	Andalusia	(Ediciones El País 2011; Moreno-Valcárcel *et al.* 2013)
2011	Sitjar	Jucar	Valencia	(Levante-EMV 2012)
2011	Sobrón	Ebro	Basque Country – Castile and Leon	(Ieltxu Vega 2011; Hay Pesca! 2012)
2012	Júcar	Jucar	Valencia	(Comunitat Valenciana 2009, 2010; Levante-EMV 2012)
2012	L' Albufera	Jucar	Valencia	(Comunitat Valenciana 2009, 2010; Las Provincias 2012; Ediciones El País 2012a b; El Mundo 2012b a)
2014	Velilla de San Antonio	Tagus	Madrid	(Hay Pesca! 2014; Ediciones El País 2016)
2014	Cuerda del Pozo	Douro	Castile and Leon	(Diario de Soria 2014; Junta de Castilla y León 2015; Tardajos de Duero 2015)
2015	García-Sola	Guadiana	Extremadura	(Navalmoral Digital 2015)
2015	Golmayo River	Douro	Castile and Leon	(Junta de Castilla y León 2015; Tardajos de Duero 2015; El Norte de Castilla 2015b)
2015	Los Rábanos	Douro	Castile and Leon	(Junta de Castilla y León 2015; Tardajos de Duero 2015; El Norte de Castilla 2015b)