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Title: New eDNA based tool applied to the specific detection and monitoring of the endangered European eel

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1 # Abstract

2 European eel (*Anguilla anguilla*) populations have declined drastically over the last
3 decades and it has been included in the IUCN red list of endangered species as critically
4 endangered since 2007. Thus, continuous population monitoring is crucial in order to
5 warrant the conservation of this emblematic species.
6 Environmental DNA (eDNA) has been recently implemented as a powerful method for
7 the detection and monitoring of freshwater species, particularly for endangerous species,
8 where eDNA methods have shown to be less invasive than other methods (e.g.
9 electrofishing).
10 In this study, we developed and validated, under controlled conditions, a new
11 species-specific tool for detecting *A. anguilla* from water samples by means of eDNA.
12 Furthermore, we applied a semi-quantitative approach to monitoring glass eel at
13 different depths (surface and bottom) during different seasons in two rivers of northern
14 Spain (Nalón and Sella rivers). We detected a significantly higher proportion of positive
15 DNA amplifications in bottom than surface samples. Moreover, the proportion of
16 replicas with positive amplification varied along the estuaries sections examined, and
17 especially in the different sampling months. The temporal detection trends found in this
18 study were compatible with the known upstream migration pattern of this species.
19 Altogether, this study contributes to the establishment of a simple, easy and
20 cheap system based on eDNA, that could be routinely applied in conservation research
21 and management programs to monitoring wild populations of endangered species.
22
23 Keywords: *Anguilla anguilla*, specific primer, conservation genetics, environmental
24 DNA
25
26

27 # 1. Introduction

28 The stocks of the catadromous genus *Anguilla* have drastically declined over the last
29 decades due to factors such as overexploitation (Bevacqua et al. 2009), illegal trade
30 (Stein et al. 2016), habitat loss and alterations in the oceans derived from
31 anthropogenic activities (Bevacqua et al. 2009). The European eel (*Anguilla anguilla*)
32 faces a high risk of extinction and it has been included in the IUCN red list of
33 endangered species as "critically endangered" since 2007 (Jacoby and Gollock 2014).
34 Due to the dramatic situation of the *A. anguilla* stocks, EU adopted regulations (No

35 1100/2007/EC) in order to reduce mortalities associated with anthropogenic factors. The
36 main aim of these regulations was to achieve an escapement to the sea of at least 40 %
37 of the silver eel biomass relative to the best estimate of escapement that would have
38 existed if no anthropogenic influences had impacted the stock (EC 2007). To ensure that
39 the regulation was implemented, each member state had to establish its own monitoring
40 methodology according to the specific characteristics of each stock.

41 In Spain, *A. anguilla* is indeed in recession as in the rest of Europe (ICES 2017b,
42 a). For accomplishing EU regulation 1100/2007/EC, electrofishing is employed to
43 monitor eel stocks (Ministerio de Medio Ambiente 2011). Although the efficiency of
44 electrofishing for counting and detecting fish has been demonstrated in shallow waters
45 (Baldwin and Aprahamian 2012), this technology is less effective in turbid and/or deep
46 waters (Knights *et al*., 2001). Consequently, data of spatial and temporal variation in
47 eel population's dynamics are often scattered and imbalanced (Jacoby et al. 2015).
48 Moreover, this technique is less efficient with small sized eels (Naismith and Knights,
49 1990), making counting of juveniles more difficult. Finally, electrofishing involves the
50 capture of wild individuals (Ellender, Becker, Weyl, & Swartz, 2012) which is
51 undesirable in the case of an endangered species such as *A. anguilla.*

52 Environmental DNA (eDNA) has arisen as a powerful method for detection of
53 marine and freshwater species. It is based on extracting DNA from environmental
54 samples like water and/or sediments and is a versatile tool that has been successfully
55 used for detection of invasive (Dejean et al. 2012), elusive (Mauvisseau et al. 2017),
56 cryptic (Janosik and Johnston 2015) and extant species (Willerslev et al. 2003). This
57 methodology has been tested in several marine and freshwater environments such as
58 deep water sediments (Corinaldesi et al. 2011), ice cores (Willerslev et al. 2007), open
59 ocean (Thomsen et al. 2012), seas (Thomsen et al. 2016), ponds (Thomsen and
60 Willerslev 2015) and rivers (Jerde et al. 2011). For fish inventory or detection, eDNA
61 based technique is less invasive than other sampling methods (*e.g.*, electrofishing
62 (Ellender et al. 2012)), since it does not involve the capture of wild individuals.
63 Therefore, the use of eDNA is less stressful for the individuals and less destructive for
64 the habitat. It works at any depth, and it is not so dependent on the turbidity of the
65 water, reducing patchy sampling and imbalance between sampling points (Jacoby et al.
66 2015). It is also worth noting the cost-effectiveness analysis of the method, as it is
67 cheaper and less time consuming than traditional methods, showing also a higher
68 detection rate (Itakura et al. 2019). It is highly reliable when placing positive (e.g. DNA

69 extraction of the target species) and negative (e.g. distilled water) controls at every stage
70 of the procedure (Smart et al. 2015).
71 The use of eDNA for the detection of anguillid species in natural environments
72 is a feasible practice. Hanfling et al. (2016) used a metabarcoding approach with
73 generalist primers that amplify the mitochondrial 12S region for tracing a depth profile
74 of the community in an English lake, and detected *A. anguilla* in the bottom. Takeuchi
75 et al. (2019b) used specific primers for the genus *Anguilla* and metabarcoding
76 technology and were able to detect several eel species from river water samples, with
77 little sampling effort and no expertise in taxonomy. Takeuchi et al. (2019a) quantified
78 the eDNA expelled by Japanese eels (*Anguilla japonica*) at different life stages in
79 controlled tanks and proved the potential of use of eDNA for detection of Japanese eel
80 during their spawning period in the ocean. Then, using a species-specific marker, *A.*
81 *japonica* DNA was detected during the spawning season in the Pacific Ocean (Takeuchi
82 et al. 2019b). Recently, the distribution and abundance of Japanese eel has been studied
83 by eDNA in Japanese rivers (Itakura et al. 2019).
84 After being transported by marine currents from the Sargasso Sea, European eel
85 larvae arrived to the continental shelf of the Atlantic coast of Europe (Miller et al.
86 2015), where they metamorphose into glass eels and colonize coastal, estuarine, and
87 river mouth habitats. Glass eels tend to be more active at night and, depending on the
88 tides, they concentrate or disperse at different depths and sides of the estuary during the
89 upstream migration phase (review in (Harrison et al. 2014)). In northern Spain, glass eel
90 density tend to be significantly greater in the deeper layer and during the new moon
91 (Aranburu et al. 2016).
92 In this study, we develop new species-specific primers for detecting *A. anguilla*
93 from water samples by means of eDNA. We further tested and validated this new
94 molecular tool in vitro, in controlled environments, and in situ from two estuaries of
95 northern Spain (Nalon and Sella rivers) using a semi-quantitative approach. For this, we
96 checked the PCR amplification in water replicates in different seasons and compared the
97 results with known patterns of glass eel abundance during the entry season, under the
98 hypothesis of detecting eel eDNA in more replicates during the peak of glass eel entry
99 than in early and late season. Altogether, this study contributes to the establishment of a
100 simple, easy and cheap system of European eel detection based on eDNA, that could be
101 routinely applied to monitoring wild European eel populations.

102

103 # 2. Methods

104

105 2.1 *Anguilla anguilla* specific marker design and laboratory validation

106 Cytochrome oxidase I (COI) gene is a widely used molecular marker for the
107 identification of species (Ratnasingham and Hebert 2007). Its mitochondrial nature
108 makes it more abundant in water samples than nuclear DNA (Ficetola et al. 2008). This
109 region is very conserved, but holds enough variability allowing sequence differentiation
110 at the species level (Xing et al. 2018).

111 Cytochrome oxidase I gene (COI) sequences of different eel species within
112 *Anguilla* genus were retrieved from GenBank database. Species-specific primers for the
113 marker region were designed using the Primer3 Online tool. The 3' end of both primers
114 were located in genomic regions exhibiting the highest difference in sequence
115 composition between European (*A. anguilla)* and American (*A. rostrata)* eels. Primer
116 specificity was checked *in silico* using the Primer BLAST online tool (https://www.
117 ncbi.nlm.nih.gov/tools/primer-blast/).

118 Primer specificity was further validated in the laboratory from cross-species
119 PCR amplifications. Samples from several species that cohabitate Spanish rivers with
120 the European eel were used for this *in vitro* test: *Salmo trutta*, *Carassius auratus,*
121 *Phoxinus sp., Squalius carolitertii, and Potamopyrgus antipodarum. A. anguilla* was
122 used as a positive control. DNA samples were extracted from ethanol-preserved
123 muscular tissue, using the DNEasy Blood and Tissue Kit (Qiagen). DNA samples were
124 quantified using Qubit High Sensitivity® fluorometer.

125 The sensitivity of primers was tested by PCR amplification on sequential
126 dilutions of *A. anguilla* DNA. Starting with a concentration of 25 ng/µl, sequential
127 dilutions were performed obtaining the following concentrations: 25, 5, 1, 0.25, 0.05,
128 0.0125, 0.0025, 0.00125, and $5*10^{-5}$ ng/µl. PCR was performed on every dilution using
129 the newly developed species-specific primers.

130

131 2.2 Primer validation on eDNA in vivo under controlled conditions

132 Species-specific primers were validated in water samples from experimental tanks with
133 known densities of adult eels. First, a set of nine 60L tanks with different numbers of
134 juvenile eels was arranged (Table 1), simulating density situations that can be found in

135 the rivers of this region (Lobon-Cervia and Iglesias 2008). The total weight of the eels
136 in each tank was recorded using an electronic scale. Individuals were kept for seven
137 days in the tanks, without feeding or water replacement, before water sampling.
138 Previous studies suggest that eDNA concentration stabilized around four days after fish
139 introduction (Maruyama et al. 2014). Then 1.5L water samples were collected from
140 each tank with sterile bottles. In addition, four 1000L aquaculture tanks of a farm
141 containing adults of European eel were sampled in order to evaluate the performance of
142 our method at high densities of eels (Table 1). Samples were kindly provided by the
143 company Marina Eel Acuicultura S.A. Water samples of 1.5L were taken with sterile
144 bottles from the border (samples "a") and centre (samples "b") of the tanks.
145 Water samples were stored at 4 °C until filtration. After the filtration step, DNA
146 extraction, quantification (see details above) and PCR amplification using the species-
147 specific primers for *A. anguilla* was performed on each sample.
148
149 2.3 Semiquantitative detection of eel in river samples
150 After validation in the lab, the *A. anguilla* specific primers were later validated in
151 southwest Bay of Biscay (Asturias region, Northern Spain). We focused on estuaries of
152 Rivers Nalón and Sella due to the fact that they host the highest population densities of
153 European eels in the region (Lobon-Cervia and Iglesias 2008). Since eel density
154 decreased upstream and showed marked seasonal and annual fluctuations in this region
155 (Lobon-Cervia and Iglesias 2008), samples were taken at different time points,
156 corresponding with the period before, during and after the peak of arrival of glass eel,
157 respectively: Autumn (November), winter (February), Spring (April) and Summer
158 (July) (Lobon-Cervia et al. 1995, Lobon-Cervia and Iglesias 2008). Therefore, the new
159 marker developed was tested in water samples from the north Spain estuary of River
160 Nalón as main case study, on November 2017, February 2018, April 2018, covering all
161 the season of glass eel entry in the river (the peak is in February). Samples were taken
162 again in July out of the upstream migration season, when glass eels are no longer in the
163 estuary and only yellow or silver eels, if any, are present there. To confirm the
164 ecological validity of the results with another river, samples were taken from River
165 Sella (200 km at east of River Nalón) in November and February. In these rivers the
166 stocks of European eel are currently monitored by electrofishing (Ministerio de Medio
167 Ambiente y Medio Rural y Marino, 2011).

168 Three 1.5L replicates were taken with sterile bottles at the bottom using niskin
169 bottles (Wurl, 2009) at eight locations evenly distributed along each river estuary
170 (Figure 1). Samplings were carried out at high tide on dates with a tidal coefficient
171 greater than 80. Although from the species ecology it is expected that eel eDNA will be
172 more abundant in the bottom, in November 2017 and February 2018 samples were
173 taken also from the river surface in the eight sampling points of River Nalón, to check if
174 eel DNA can be also detected from the surface.
175 All water samples were immediately transported to the laboratory in coolers. Upon
176 arrival, the water samples were stored at 4 °C and immediately filtrated following the
177 procedure described next.
178
179 2.4 Environmental DNA (eDNA) extraction
180 Water samples were filtered using an Acetate cellulose membrane (Fisher Scientific) of
181 0.22 μm pore size and a filter holder. Filtration took place inside a laminar flow cabinet
182 previously treated with UV light to avoid any contamination. The filter holder was
183 dismantled, cleaned with 50 % bleach, rinsed with distilled water and treated with UV
184 for 20 minutes before use and between samples. A negative control consisting of 1 L of
185 milliQ water filtrated between two real samples was included in all the analysis. Filters
186 were stored at -20°C until extraction.
187 All the collection, filtration, extraction and analysis process were performed
188 following the recommendations from Goldberg et al. (2016) in order to avoid any cross
189 contamination in the different steps. DNA from 1.5 L water samples was extracted with
190 the PowerWater® DNA Isolation Kit (Mobio laboratories) following the manufacture's
191 protocol. In addition, the whole extraction process was carried out inside a laminar flow
192 cabinet. Additionally, negative controls were included in each extraction and in all
193 posterior PCRs amplifications; consisting of a negative control for filtration (previously
194 described) and a negative control for extraction which consisted in a clean membrane.
195 All the pre-PCR steps were done inside the laminar flow cabinet after 20 minutes of UV
196 light decontamination, and the post-PCR steps were done in a separate laboratory unit.
197 Finally, total DNA on each water sample was quantified using Qubit High Sensitivity®.
198
199 2.5 PCR amplifications

200 All PCR amplification reactions were performed in a total volume of 20 µl, including
201 Green GoTaq R® Buffer in 1X concentration, 2.5 mM $MgCl_2$, 0.25 mM dNTPs, 1 µM
202 of both forward and reverse primers, 4 µl of template DNA, 200 ng/µl of bovine serum
203 albumin (BSA) and 0.65 U of DNA Taq polymerase (Promega®). Cycling conditions
204 were 95 °C for 5 min initial denaturation, then 40 cycles of 95 °C for 30 s, 65 °C for 30
205 s, and 72 °C for 30 s, followed by a final extension at 72 °C for 7 min.
206 The PCR product of a positive amplification from Nalón river water was
207 sequenced in order to confirm the identity of the band. For this, the 110 bp band was
208 excised from the agarose gel and purified with QIAquick Gel Extraction Kit®. After
209 purification, the sample was sequenced by Sanger method, manually edited using
210 Bioedit® and searched in databases using BLAST. In addition, the environmental
211 samples that did not amplify with the species-specific primers were later PCR-amplified
212 using generalist primers (Leray et al. 2019) in order to discard the presence of inhibitors
213 (false negatives). The appearance of an amplicon of 500 bp that is the expected
214 fragment size amplified from Leray et al. (2019) primers was considered as proof of the
215 lack of inhibitors in the sample.
216 All PCR procedures were carried out under sterile conditions in a laminar flow
217 hood. All PCRs counted with a positive control with *A. anguilla* DNA and a negative
218 control with water instead of eDNA. All PCR amplifications were performed in
219 GeneAmp 2700® termocyclers and visualized in 2.2% agarose gels.
220

221 2.6 Statistical analyses
222 All statistics and plots were performed in R 3.6.1. For semi-quantitative approach in
223 river samples, the variable employed was the proportion of positive PCR amplification
224 over the total number of replicates in each sampling point and time. From three
225 replicates this variable can take four values. The effect of the spatial location (upstream
226 versus downstream as levels), river (Nalón versus Sella as levels) and sampling season
227 (sampling months as levels) were tested using ANOVA methodology, after checking for
228 dataset homoscedasticity and normality with Breusch-Pagan and Shapiro-Wilk tests,
229 respectively.
230

231 3. Results

232 3.1 The new marker

233 The newly designed species-specific primers amplified a 110 bp long region within the
234 mitochondrial COI gene of *A. anguilla*. This region is located between the positions
235 6075 and 6185 of the mitochondrial genome (Accession number: NC_006531.1).
236 The species-specific primers designed *in silico* were:
237 Forward: Ang-COI_F: 5'-GCTGTATTAGTAACCGCCGTTTT-3'
238 Reverse: Ang-COI_R: 5'-GCAGGATCAAAGAAGGTCGT-3'
239

240 3.2 Marker validation

241 *In silico* we confirmed that primers amplified only the target region. A search on the
242 GenBank database using BLAST gave significant matches only with *A. anguilla* COI
243 mitochondrial region (single COI sequences or within the whole mitochondrial DNA
244 sequence). All matches showed 100 % identity and query coverage.
245 The sensitivity test showed amplification at all the concentrations, even at the 1:500,000
246 dilution corresponding to $5x10^{-5}$ ng/µl of DNA (Figure 2A). The cross-species
247 amplification test showed a clear 110 bp band only for *A. anguilla* (Figure 2B), not for
248 any other species assayed. A weak band appeared in *Phoxinus* sp. sample, but this band
249 was lighter than 110 bp and of much lesser intensity.
250 In the validation in vivo, all the samples taken from tanks with low densities of
251 eels showed successful amplification, as could be expected from the high sensitivity of
252 this marker in vitro (Table 1; Supplementary Figure 1, below). A stronger amplification
253 can be seen with increasing concentrations of DNA. Indeed, in the eight samples taken
254 from aquaculture tanks with high density of eels provided positive amplification with
255 the new primers (Supplementary Figure 1, above).
256

257 3.3 Field validation

258 Environmental DNA was obtained from all the estuary samples. A random set of
259 samples from November and February was chosen for a quantification with Qubit High
260 Sensitivity® All samples showed a similar amount of total eDNA with a mean of 2.19 ±
261 0.32 ng/µl (Supplementary Table 1). Only one sample (point 8, November) was below
262 the quantification threshold using this methodology but showed positive amplification
263 with the species-specific primers anyway. This suggests that low amount of eDNA in
264 river water samples is not a problem for the application of this sensitive marker.
265 Many positive PCR amplifications were obtained with the new eel-specific
266 markers from eDNA estuary samples (Supplementary Table 2). In order to validate the

267 specific amplifications, samples that did not show amplification were tested for PCR
268 success using different primers to discard false negatives. All the negative
269 amplifications with species-specific primers showed positive amplification with Leray
270 et al. (2019) primers, suggesting lack of inhibition for all the samples tested. To check
271 possible false positives due to cross amplification with species not assayed in *in vitro*
272 tests, randomly selected positive amplifications were extracted from the agarose gel and
273 sequenced. After manual editing with BIOEDIT the sequence identity was assigned
274 using online BLAST tool against GenBank database. The top 50 match hits
275 corresponded to *A. anguilla* sequences with an e-value of $6x10^{-6}$ and only two matches
276 happened with *A. japonica* in the top 100 results, something that can be explained from
277 the short amplicon length and some intraspecific variation in these species. These
278 results confirm the specificity of the marker designed.

279 In River Nalón case study, the proportion of replicas with positive amplification
280 varied along the estuary section examined and especially in the different sampling
281 months (Figure 3). *A. anguilla* eDNA was detected all the months only in the upstream
282 sampling point, while detection in all the sampling points occurred only in February
283 corresponding with the peak of glass eel entry. Out of the entry season, i.e. in July in
284 this study, European eel eDNA could only be detected upstream. The spatial patterns
285 found in early and late entry season (November and April respectively) were quite
286 similar, not detecting eel eDNA in the points #4 and #7. According to the expectation of
287 significant change along the season of upstream migration of glass eels, two-way
288 ANOVA without replication was highly significant for the temporal variation ($F_{3,21} =$
289 26.19, $p << 0.001$), while spatial variation was not significant (Supplementary Table
290 3A).

291 Regarding the difference between water column depths in the detection of eel
292 DNA, first total eDNA was quantified. Results showed that surface samples contained
293 four times less DNA concentration than the bottom ones (0.4 ± 0.05 and 1.7 ± 0.32
294 ng/µl, respectively) (Supplementary Table 1). Although total eDNA also includes DNA
295 from other species, the results were consistent with amplification of eel eDNA from
296 more locations and replicates in bottom than in surface waters. In River Nalón, 87% of
297 the samples taken at the bottom showed positive DNA amplification while the
298 proportion was 62% in surface samples. However, the number of positive amplifications
299 per sampling point in surface and bottom samples (Figure 4A) were highly significantly
300 correlated (Spearman's rs = 0.779, 14 d.f., p = 0.0003).

301 In River Sella the results obtained in November and February were generally
302 consistent with the results obtained from River Nalón. More positive amplifications and
303 from more sampling points were found in February, the river entry peak for glass eels in
304 the region, than in November. Two-way ANOVA confirmed the results were not
305 significantly different between rivers, and the significant temporal variation of the
306 proportion of replicas with positive PCR amplification, higher in February than in
307 November in the two rivers ($F_{1,28} = 5.366$, $P = 0.028$; Supplementary Table 3B).
308

309 Discussion

310 In this study, we describe reliable species-specific primers for the detection of *A.*
311 *anguilla* in environmental water samples from north Spanish estuaries. The new marker
312 is highly sensitive. Semi-quantitative estimation of eel eDNA during and after the
313 period of upstream migration of glass eels in two different estuaries confirmed the
314 expected hypothesis of more positive water replicas during the peak of entrance in the
315 river. All together these results allow us to introduce this new marker based on
316 environmental DNA as a powerful tool for helping in the control and monitoring of
317 European eel populations in the wild.
318 To our knowledge, this is the first eDNA tool validated in the field for detection
319 of *Anguilla anguilla* from water samples in European rivers. Species-specific primers
320 have been designed for identification of *A. anguilla* tissue DNA (Trautner 2013) and for
321 identification and quantification of *A. japonica* in water samples (Takeuchi et al.
322 2019a), but not for the European eel yet. For the endangered conservation status of this
323 species, this new tool opens new opportunities for its management such as monitoring
324 in the field without disturbing the community with electrofishing. In Japanese rivers,
325 (Itakura et al. 2019) found that electrofishing detected Japanese eel in 30% less sites
326 than eDNA, suggesting that species-specific primers allowed the detection of eels from
327 eDNA with a better efficiency than electrofishing.
328 On the technical side, this new tool seems to be sensitive and species specific. *In*
329 *silico*, it matches significantly only with the species *A. anguilla*, and, in the two north
330 Spanish rivers assayed, only amplifies *A. anguilla* DNA. These new primers were
331 highly sensitive, compared to previous studies (Trautner 2013), since they amplify even
332 from very diluted DNA samples. The quantity of DNA extracted directly from tissue is
333 much higher than in environmental samples (Takahara et al. 2012); moreover, a big
334 quantity of eDNA could be bacteria, especially in culture tanks where they proliferate

335 easily (Callol et al., 2015; Alcaide, Blasco, and Esteve, 2005). Notwithstanding it,
336 positive amplification was found in all the experimental tanks, even at a concentration
337 as low as 0.75 ng/µl of eDNA (Tank #9). This confirms the sensitivity of the new tool.

338 In the field, our results support the idea of sampling water from the river bottom.
339 Samples taken at the bottom have a higher chance of detection and are more
340 representative than surface samples. Samples taken at the surface may underestimate eel
341 presence as glass eels migrate near the seabed (Yokouchi et al. 2009). In general all eel
342 behaviour occurs at the bottom of the rivers (McCarthy et al. 2008), and detection by
343 eDNA is less probable with increasing distance from the source (Murakami et al. 2019,
344 Stewart 2019). Quantifications and inhibitor tests suggest that PCR amplification
345 failures were most likely due to lack of enough *A. anguilla* DNA and not to the presence
346 of inhibitors. Samples were taken near the riverbed, where humic acids from vegetal
347 biomass degradation (typical PCR inhibitors in river water) are less likely to be at high
348 concentrations (Jane et al. 2015).

349 Repeat sampling for eDNA has been successfully used to document the arrival
350 of migratory species or assess the success of habitat restoration in other endangered fish
351 such as chinook salmon (Laramie et al. 2015). Regarding temporal detection trends, our
352 results were fully compatible with the known upstream migration pattern of this species.
353 After crossing the Atlantic Ocean, the entrance of juveniles in the estuaries occurs
354 gradually and catches of glass eel per unit of effort increase during the winter. At the
355 latitude of our sampling points the peak of entry is in February-March (Righton et al.
356 2016, Stratoudakis et al. 2018). Our results with a greater proportion of positive
357 amplifications in February samples in the two estuaries are in concordance with the
358 migratory behaviour of European eel.

359 Some slight differences among sampling points in the detection of eels could be
360 explained from their migratory behaviour. When glass eels arrive from the Sargasso sea,
361 they are known to stay longer at the mouth of the river than in the estuary itself
362 (Harrison et al. 2014). This could explain the higher detection on point 8 which
363 represents a transition point between the estuary and open sea waters. The higher
364 detection on the top of the estuary could be due to the narrowing of the river at that
365 point, which increases individuals per unit of water, and perhaps also to the presence of
366 adult eels that would explain positive results in July.

367 As a final remark, these new primers are ready to use in environmental samples.
368 They have shown its specificity and sensitivity in all the tests performed and are

369 therefore a reliable tool for detection of *A. anguilla*. These primers can also be used in
370 other geographical areas with minimal further development, just checking for the
371 absence of cross-amplification with local species. Another powerful application of
372 eDNA methods is to estimate population abundance from the concentration of eDNA in
373 water samples (Takahara et al. 2012, Goldberg et al. 2015). Further studies are required
374 to adapt and validate the proposed marker towards its application for the quantification
375 of European eel population abundance based on environmental samples.
376

377 Conclusions

378 The primers designed in this study are a sensitive tool for the detection of *A. anguilla*
379 from environmental water samples. From the biology of the species bottom water
380 sampling would be recommended. Altogether, the methodology employed in this study
381 can be easily applied in conservation projects for monitoring endangered populations
382 such as European eel, being a complementary or alternative method to electrofishing
383 techniques.
384
385
386 Data accessibility
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388 Data availability
389 All the data employed in this study will be available upon approval
390

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533 **TABLES AND FIGURES**

534 **Table 1.** Experimental validation of the new marker from eDNA under controlled eel
535 densities in experimental tanks. Tank volume, number of individuals (N), estimated
536 biomass (g), eDNA quantification (ng/µL). Positive PCR amplification with the new
537 primers occurred from all the tanks.

Tank	Tank volume	N	Biomass (g)	eDNA (ng/µL)	Marker amplification
1	60L	4	80.57	3	Yes
2	60L	4	81.15	1.25	Yes
3	60L	8	196.30	7.5	Yes
4	60L	14	211.70	4	Yes
5	60L	24	399.50	7.5	Yes
6	60L	20	358.60	6	Yes
7	60L	43	748.60	15	Yes
8	60L	43	745.50	12.6	Yes
9	60L	3	34.50	0.75	Yes
1a-1b	1000L	108	17444	2900-2700	Yes
2a-2b	1000L	121	21894	2170-1920	Yes
3a-3b	1000L	284	40750	5920-7340	Yes
4a-4b	1000L	207	30539	2900-2080	Yes

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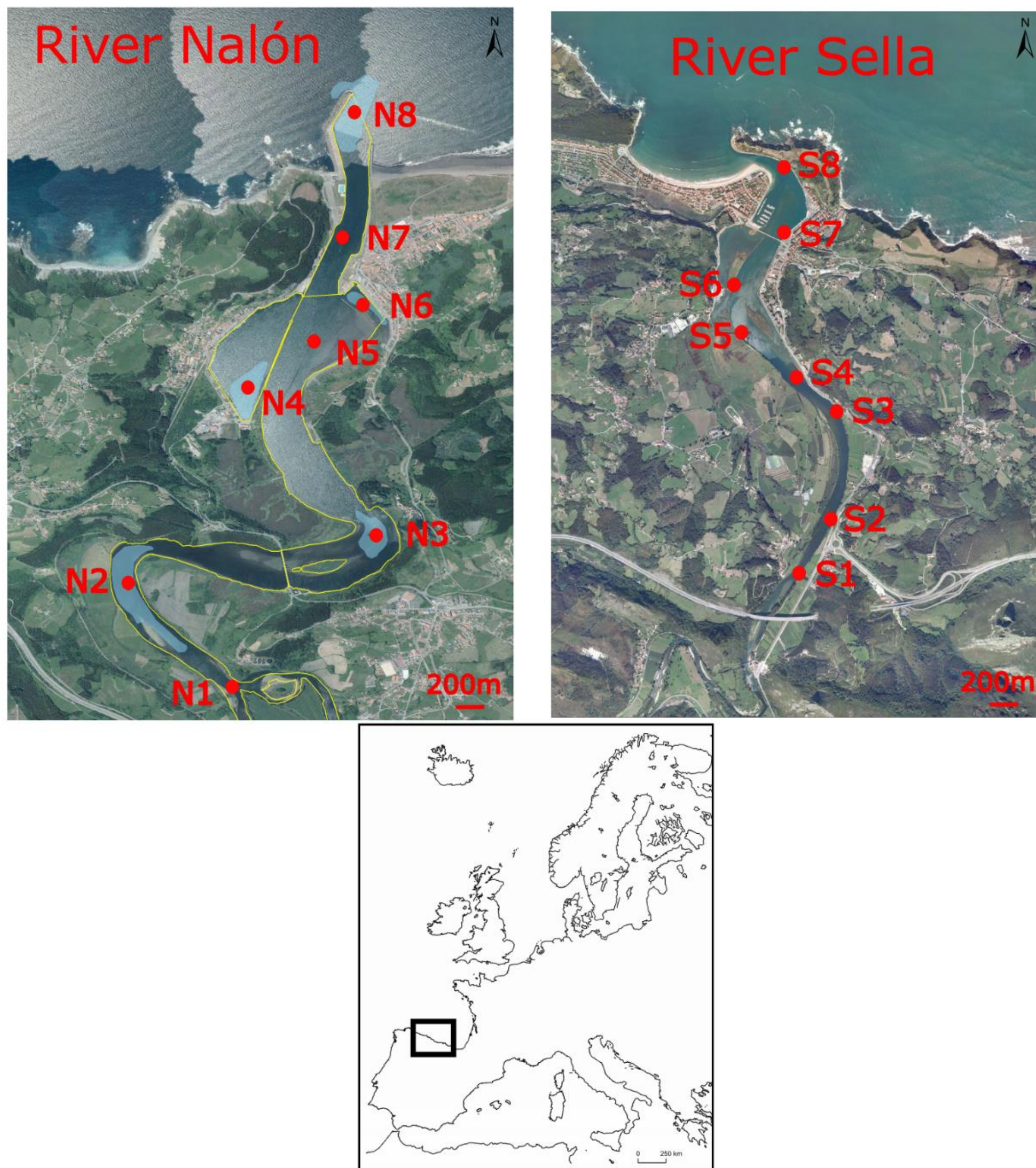
542 **Figure 1.** Map showing the eight sampling localities within river Nalón & Sella
543 (Asturias, Spain).



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548 **Figure 2.** Agarose gels *showing PCR amplicons with species-specific primers*. (A)
549 Sensitivity test performed on serial dilutions of *Anguilla anguilla* DNA. Ratio of
550 dilution shown over every sample from an initial 1:1 concentration of 25 ng/µl. (B)
551 Cross-species amplification test. (1) *Salmo trutta*; (2) *Carassius auratus*; (3) *Phoxinus*
552 sp; (4) *Squalius carolitertii*; (5) *Potamopyrgus antipodarum*; (6 and 7) *A. anguilla*.
553 Marker 100-1000bp (M) and negative control for amplification ("-") are shown in both
554 gels.
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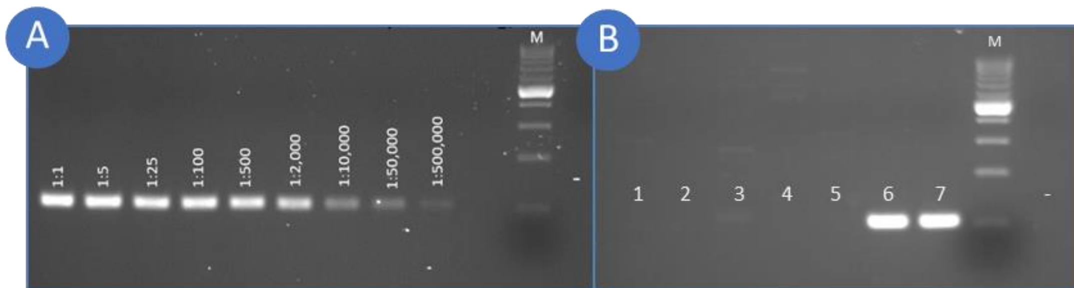

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559 **Figure 3.** Proportion of positive PCR amplifications with the new primers, per sampling

560 point and month in River Nalón.

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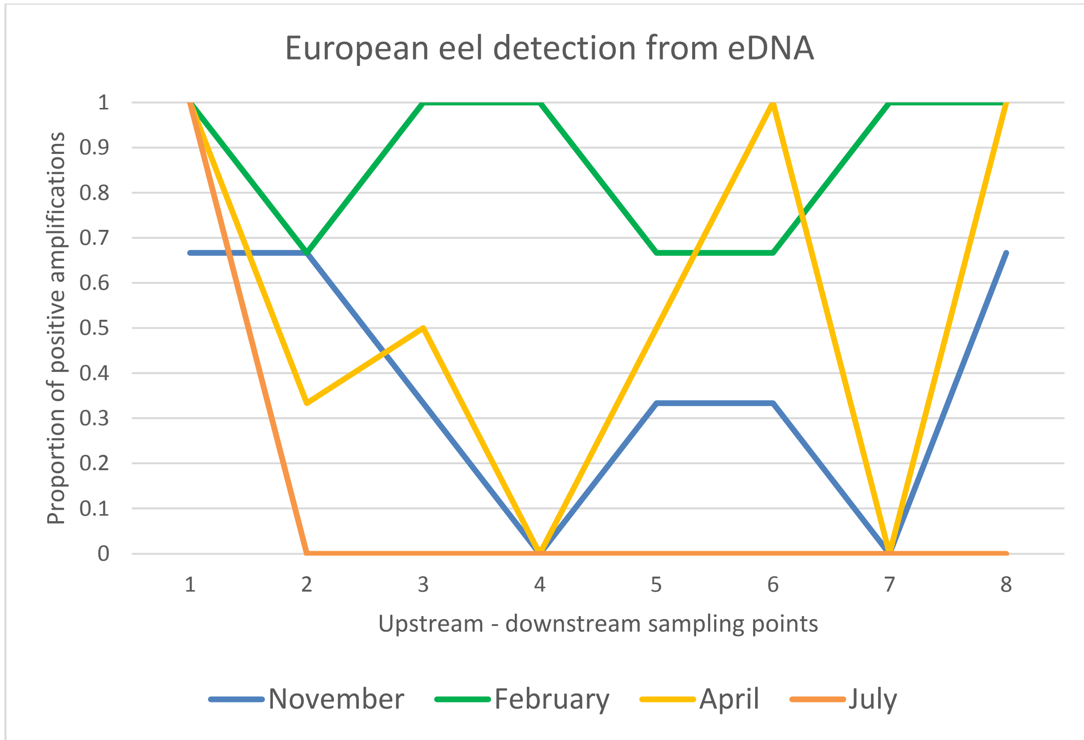

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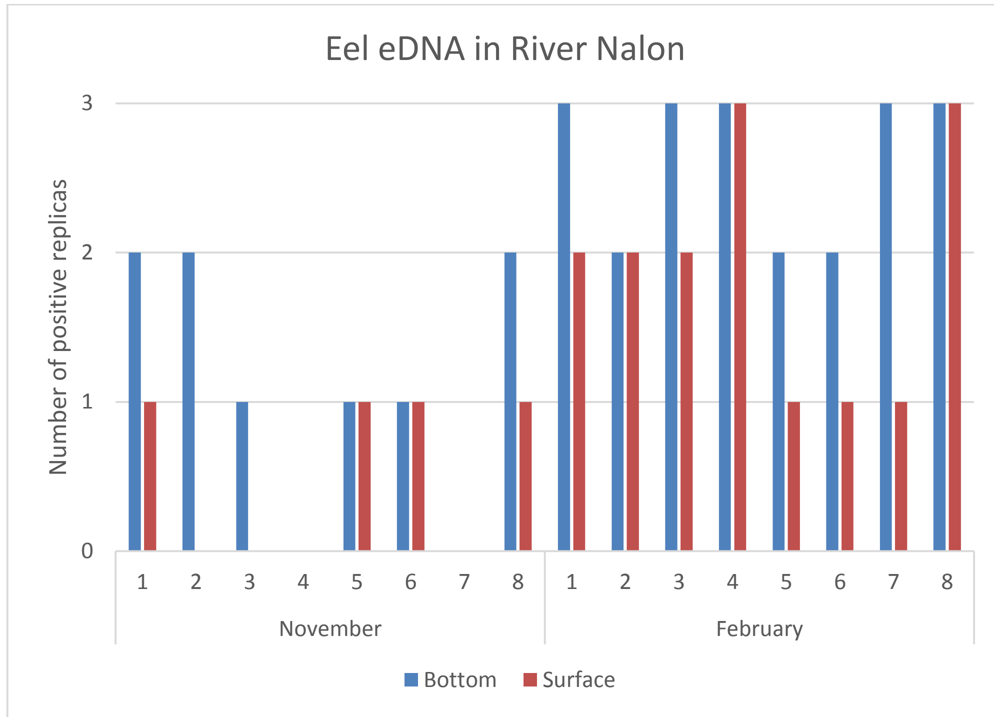
564 **Figure 4.** Number of positive PCR amplifications with the new primer obtained per
565 sampling point from bottom and surface samples in River Nalón (A), and from bottom
566 samples in River Sella (B), in November 2017 and February 2018.

567 A)



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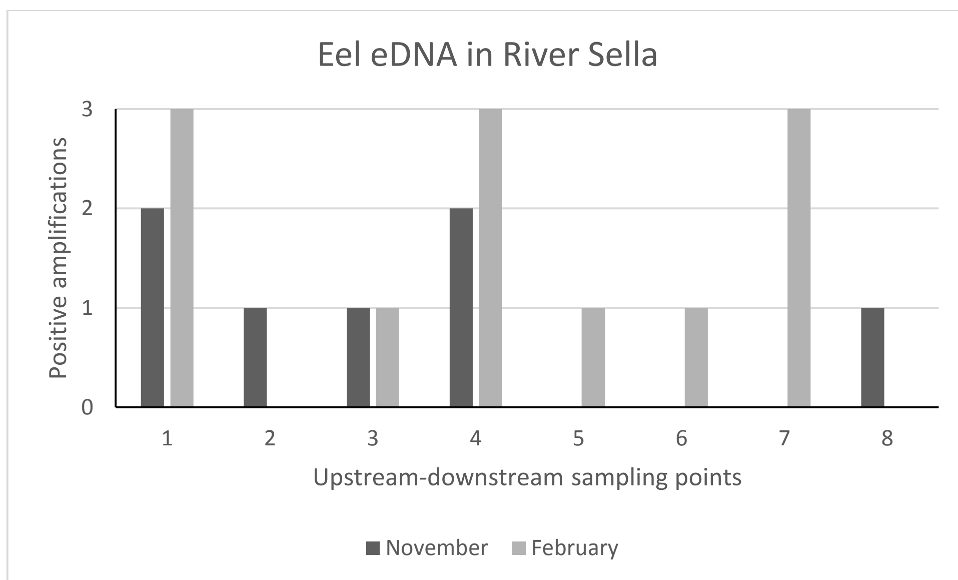
569 B)



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Javier Burgoa Cardás: Conceptualization, Methodology, Formal analysis, Writing - original draft. **Dumas Deconinck:** Methodology, review & editing. **Isabel Márquez:** Methodology, review & editing. **Paloma Peón Torre:** Methodology, review & editing. **Eva Garcia-Vazquez:** Conceptualization, Formal analysis, Writing - review & editing, Funding acquisition. **Gonzalo Machado-Schiaffino:** Conceptualization, Methodology, Writing - review & editing, Supervision, Project administration, Funding acquisition.

Supplementary Files

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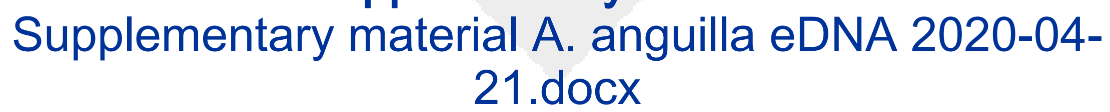
Supplementary Material



Supplementary material A. anguilla eDNA 2020-04-21.docx