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First application of environmental eDNA for detecting the presence of the European eel [*Anguilla anguilla*, (Linnaeus, 1758)] in the Adriatic, as a basis for conservation remarks

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Abstract

Anguilla anguilla, the European eel, is an important species for aquaculture and fisheries. Its population has dropped dramatically in recent decades, reaching an all-time low. As a result, it has been listed as critically endangered on the IUCN Red List of Endangered Species since 2007. Therefore, constant population monitoring is essential to ensure the survival of this iconic species. Glass eel recruitment is declining worldwide, including the populations in the Mediterranean region. Despite the negative impact of man-made activities in Mediterranean coastal waters over the past few decades, data on spawning biomass escaping from the Mediterranean highlights the region's importance for the global eel supply. Eel research and monitoring is done using conventional techniques, which have certain drawbacks. Therefore, the use of molecular-based detection as a credible choice for monitoring species in aquatic ecosystems was recently shown to be an effective management plan alternative. We present the first use of environmental DNA for monitoring eel populations in the Adriatic Sea and in the complex Dinaric karst freshwater ecosystem. The method has been demonstrated to be accurate and useful for detecting the presence of *A. anguilla* eDNA and identifying conservation areas. This is also the first study investigating the range and presence of the European eel in the Adriatic and in the Mediterranean Sea, as well as in underground karst systems, springs, and in the karst poljes of the Eastern Adriatic coast.

Keywords: eels; *Anguilla*; environmental monitoring; Mediterranean region; environmental DNA; conservation genetics.

Introduction

The European eel, *Anguilla anguilla*, (Linnaeus, 1758), is a catadromous fish made up of a single mating population that spawns in the Sargasso Sea (Cresci, 2020). Eels migrate from the Atlantic Ocean as larvae and reach Europe's continental slope, where they transform into post-larval glass eels. The latter make it to the continent, where some enter fresh water, others stay in the marine environment, and still others move between the two (Cresci, 2020). In Europe, eels inhabit the area from Norway to the southern parts of the Mediterranean Sea (Dekker, 2000; 2003). Historically, the European eel was, and still is, an important species in aquaculture and fisheries (Cresci, 2020). This species is one of the most important commercial fish in the world (Violi *et al*., 2015), especially since the demand for glass eels from eel farms in Asia drives the glass eel trade. Due to the fact that Japanese glass eels (*Anguilla japonica*, Temminck & Schlegel, 1846) were in short supply in the 1990s, European glass eels were frequently used as a substitute (Stein *et al*., 2016). Unfortunately, eel stocks have reached their all-time low and concerns regarding the state of the stock have been highlighted by a drop in captures of this species at all stages (Capoccioni *et al*., 2020). Over the last three decades, glass eel recruitment has plummeted to 10% of what it was in the 1960s and 1970s (Dekker, 2016). The main cause of decline of *A. anguilla* populations is the combination of natural and anthropogenic causes, such as uncontrolled exploitation, illegal trade, habitat alterations, and habitat loss due to human activities, contamination, and diseases. These impacts act together, affecting all developmental stages of the European eel, leading to decreased biomass of all stocks (Dekker & Beaulaton, 2016; Miller *et al*., 2016; Bevacqua *et al*., 2009; Stein *et al*., 2016; Jacoby *et al.,* 2015). For that reason, the Euro-

pean Union adopted an eel protection and recovery plan in 2007 (Anonymous, 2007). This regulation required the EU Member States to adopt national Eel Management Plans (EMPs) by 2009, with the goal of reducing anthropogenic mortality and restoring a spawner run. As a result, in nineteen EU countries, national management plans have been formed, preventive measures have been adopted, and additional information on the stock's status has been collated (Dekker, 2016; Dekker & Beaulaton, 2016). Furthermore, in September of 2007, *A. anguilla* was listed in Appendix II of the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES), imposing regulation on its international trade. Still, the European eel remains a critically endangered species according to the last IUCN assessment (Pike *et al*., 2020).

As evidenced by a concurrent decline in glass eel recruitment, including local stocks in the Mediterranean region (Aalto *et al*., 2016; Amilhat *et al*., 2014), the decline of the global eel stock affects the entire geographical range of the European eel, including the southern part of its distribution area. The eels are largely associated with coastal lagoons in the Mediterranean region (Nielsen & Prouzet, 2008), areas which encompass a total surface area of 5800 km^2 and account for a substantial fraction of the entire continental eel habitat (Cataudella *et al*., 2014; Capoccioni *et al*., 2020). A steep decrease of eel productivity in these coastal lagoons is indicative of significant ongoing negative changes in the quality of these habitats. Several man-made activities have impacted the Mediterranean coastal lagoons throughout the last several decades, resulting in increased eutrophication and pollution of these shallow water habitats with pesticides and pharmaceutical loads (Parolini *et al*., 2010; Pinto *et al*., 2016; Riascos-Flores *et al.*, 2021). This may have influenced the reproductive potential of the Mediterranean eel stocks. Strong evidence supports chemical pollution being one of the main reasons behind the sharp decline in recruitment and abundance of the European eel (Belpaire *et al.*, 2019). Pesticides and pharmaceuticals are well known to be a source of xenoestrogens. Xenoestrogens interfere with the natural functions of estrogens and induce reproductive issues, such as reduced sperm count in males and reduced fecundity and egg hatchability in females (Badamasi *et al.*, 2020). Studies suggest that roughly 35% of the healthy spawning biomass is still escaping from Mediterranean lagoons (Aalto *et al*., 2016), which emphasizes their importance in contributing to the world eel supply (Capoccioni *et al*., 2020).

Currently, research and monitoring of eels relies on traditional methods like electrofishing and trapping, which have certain limitations. These methods are increasingly controversial because they are recognized to be non-selective (with certain non-target species captured) and very disruptive for the ecosystem, sometimes resulting in the death of certain specimens (Robinson *et al.*, 2018; Wang *et al.,* 2021). Though some research requires capturing individual specimens using traditional methods (i.e*.,* monitoring the population density or population genetics studies), problems still exist, particularly in the context of monitoring protected species such as the European eel and aiming to determine only species presence/absence. As a result, these traditional methods tend to be replaced by molecular methods, which have been widely developed over the past ten years (Thomsen & Willerslev, 2015). This method is commonly called environmental DNA (eDNA) and allows the detection of an organism's DNA which comes from various tissues, e.g., skin, eggs, and mucus, shed in its surroundings, without the requirement to see the target species at any stage of its life (Ficetola *et al*., 2008). The effectiveness of eDNA-based detection techniques has been proven for the early detection of rare, endangered, and endemic organisms (Piggott*,* 2016), as well as for non-native and invasive species (Baudry *et al*., 2021, Dubreuil *et al.,* 2022). This strategy has also been demonstrated as a sustainable practice for anguillid species in their native habitats, making it a valuable technique for research and conservation (Hänfling *et al*., 2016; Weldon *et al*., 2020; Burgoa Cardás *et al*., 2020).

In the Croatian part of the Adriatic Sea drainage system, the European eel occurs in all rivers of the basin (Milošević *et al*., 2021). Based on data provided by the Croatian Institute for Biodiversity, records indicate a wide distribution of *A. anguilla* in the Croatian coast of the Adriatic Sea during the last 120 years (Fig. 1). It is interesting and important to notice that the European eel is not only present in marine or freshwater habitats in the Adriatic region, it is also frequently detected in lakes and rivers more than 100 kilometers inland. Moreover, the eel was also detected in different karst fields (poljes) like in Gacko, Ličko, Imotsko, Vrgoračko, and Konavosko polje.

Large populations of this species have been recorded in the lower parts and estuaries of large rivers (Neretva, Zrmanja, Krka, Cetina, Jadro, Žrnovnica, etc.), and in lakes (Vrana Lake near Biograd, Baćina Lakes near Neretva River, Vrana Lake on Cres, etc.) (Piria *et al*., 2014; Dulčić & Glamuzina, 2006). Zrmanja and Neretva rivers are large freshwater systems that drain into the Adriatic Sea (Bonacci, 1999; Riđanović *et al*., 2010). Estuaries of both rivers are areas of high biodiversity, especially for the freshwater, brackish, and marine ichthyofauna (Mrakovčić *et al*., 2006; Glamuzina & Dobroslavić, 2020). Large populations of *A. anguilla* can be found in both rivers, making them important habitats for this species in the Adriatic Basin. Moreover, the European eel is one of the most dominant species in the Neretva River, with 3.75% of the biomass share (Glamuzina & Dobroslavić, 2020). The existence of eel populations on Pag, Ugljan, and Pašman Islands is historically known, especially in the larger bodies of water (e.g*.,* lakes Kolansko Blato, Velo, and Malo Blato on Pag Island). Interestingly, even though A. *anguilla* is not considered a stygophilic species, it has also been detected in the underground habitats of the Dinaric karst. Underground eel migrations were already mentioned in the early 20th century with the detection of eels in karst poljes (Ćurčić, 1916). Research conducted on the Timavo River (Slovenia, Italy) confirmed the underground migrations of eels. *A. anguilla* was also detected in other karst poljes – Mostarsko blato

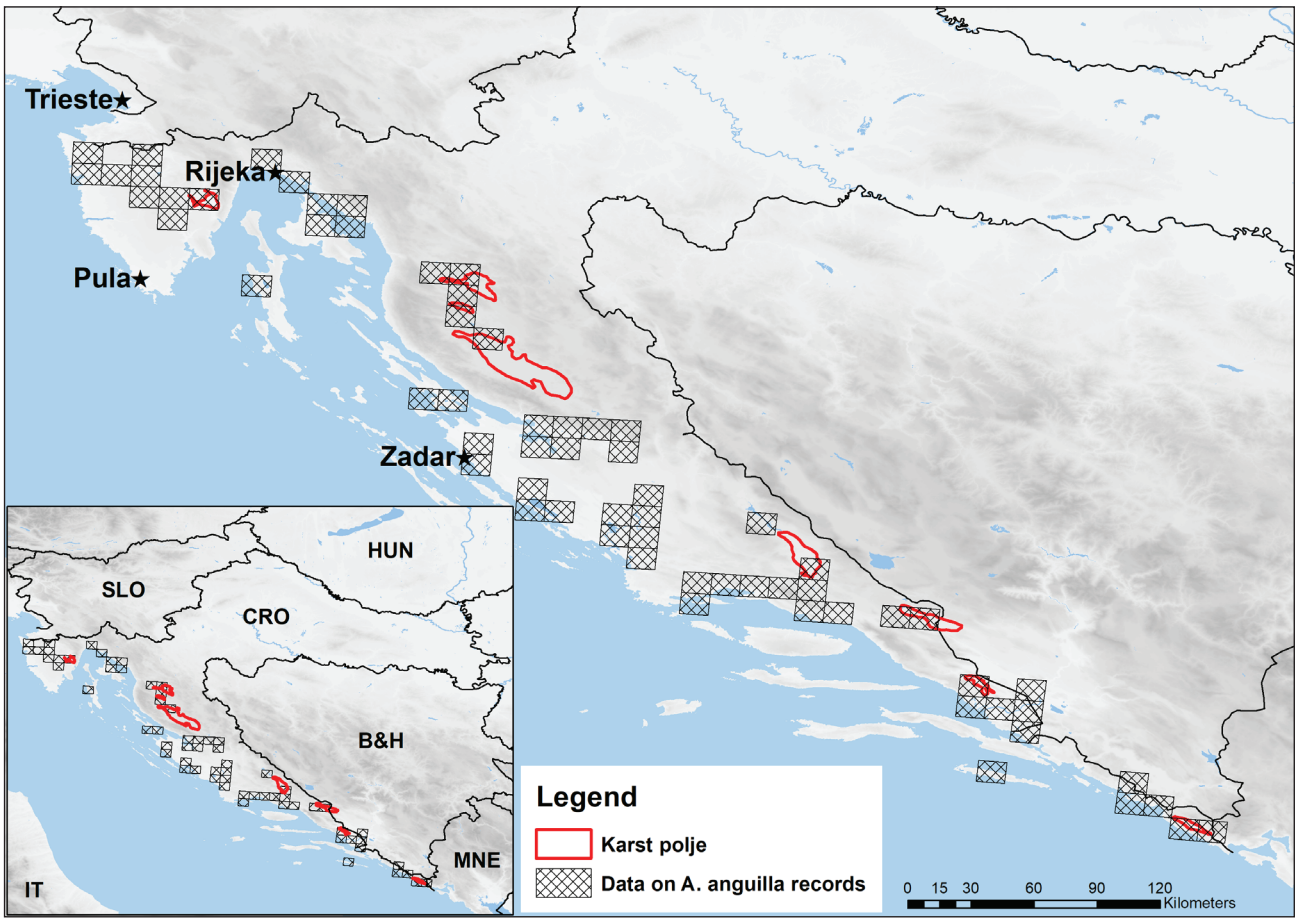

Fig. 1: Literature data on *A. anguilla* presence in the last 120 years in the Croatian Adriatic.

(230 m.a.s.l.), Imotsko blato (150 m.a.s.l), Popovo polje (300 m.a.s.l.) (Ćurčić, 1916; Dojmi, 1939).

At present, despite its critically endangered species status, the efforts for its protection, as well as its commercial importance, surprisingly little effort was put into studying the ecology and distribution of *A. anguilla* in Croatia and in the Adriatic region in general. Most of the published research has been focused on the length-weight relationship (e.g., Dulčić & Glamuzina, 2006; Piria *et al*., 2014; Castadelli *et al*., 2014), otoliths (Kanjuh *et al*., 2018; Milošević *et al*., 2021), parasites (Di Cave *et al*., 2001; Dezfuli *et al*., 2014), or toxicology (Storelli *et al*., 2007). The majority of data on the presence of *A. anguilla* in the Croatian Adriatic is collected from the studies which aim at other freshwater or marine species, where the eel was detected as a bycatch.

The study we present here is the first eDNA study conducted in the broader Adriatic region, with the aim of monitoring eel populations in this area of major ecological importance. Our field sampling was carried out with consideration of the historical data on the presence of the European eel, and our aim was to be as exhaustive as possible. The laboratory protocol was optimized, following the scale of Thalinger *et al.* (2021), resulting in robust, specific, and highly sensitive results for our target species. In this study, we report the first data on the presence of European eel DNA, thanks to an effective, non-disruptive, and reproducible method, in the context of future monitoring studies of these endangered eel populations.

Materials and Methods

Sampling sites

Sampling was conducted during ten days in September of 2021 on islands and one week in December of 2021 in the Neretva River delta. It included a total of 24 locations - fifteen on the four islands of the Zadar County (Pag, Ugljan, Pašman, Dugi otok), and nine localities in the Neretva River Delta (Table 1, Fig. 2). The sampling localities were selected considering the available literature data on *A. anguilla* in Croatia, the existing data on wet habitats on the chosen islands, unpublished data, and the habitats that correspond to the ecology of the target species.

Sample collection

To avoid potential field cross-contamination, filter housing and tubing were totally disinfected in a 10% bleach (sodium hypochlorite) solution for 20 minutes after each filtration step, then transferred and thoroughly rinsed using tap water to eliminate the bleach. Though tap water is not sterile, it can be assumed that it is completely free from eel DNA, single-targeted species in this research, thus not affecting possible cross-site contamination.

After collecting water from the water bodies, filtration took place on-site using an electric vacuum pump

Table 1. Eel sampling locations, positive or negative detection, and habitat type. Island locations are marked with grey cells (1-15); and the rest are locations from the Neretva River Delta (16-24).

No.	Locality	Coordinates		Habitat type	Detection	Proportion of qPCR positive replicates	Mean Ct Values ± SD
		Longitude	Latitude				
1	Dugi otok, Velo jezero	15.10932	43.941757	Freshwater	-	0	-
2	Dugi otok, pond	15.10992	43.962694	Freshwater	-	0	-
3	Pašman, Jelenići	15.237878	44.015086	Marine	-	0	-
4	Pašman, Barotul	15.362688	43.964398	Marine	+	1	31.94 ± 0.49
5	Ugljan, M. Lukoran	15.165024	44.096911	Brackish	+	1	30.54 ± 0.45
6	Ugljan, pond in the Vela Lamjana	15.197886	44.051487	Brackish	-	0	-
7	Ugljan, Vela Lamjana	15.198543	44.05038	Marine	+	0.67	35.32 ± 0.76
8	Pag, Solana Pag (saltern)	15.07988	44.416991	Marine, hypersaline	+	0.67	35.3 ± 0.66
9	Dugi otok, pond Dugo polje	15.117474	43.93644	Freshwater	-	0	-
10	Dugi otok, lake Mir	15.162003	43.889349	Marine, hypersaline	-	0	-
11	Dugi otok, Malo jezero	15.101682	43.948398	Freshwater	-	0	-
12	Pag, Velo Blato	15.151155	44.351151	Slightly brackish	-	0	-
13	Pag, Sega lagoon	15.096664	44.355539	Marine	+	1	28.9 ± 0.54
14	Pag, Malo blato mouth	15.114092	44.369691	Marine	-	0	-
15	Pag, Kolansko blato	14.918247	44.514459	Brackish	+	1	33.26 ± 0.49
16	Mliništa	17.615854	42.992206	Freshwater	+	1	31.21 ± 0.8
17	Podolac	17.658710	43.046335	Freshwater	+	1	33.95 ± 1.09
18	Bađula, karst spring	17.610419	42.962383	Freshwater, spring	+	0.89	34.34 ± 0.71
19	Bijeli Vir	17.653863	43.012689	Freshwater	-	0	-
20	Sv. Mihovil	17.629369	43.000556	Freshwater	+	1	33.47 ± 0.67
21	Bijeli Vir, karst spring	17.654834	43.010184	Freshwater, spring	+	0.67	34.68 ± 1.84
22	Glušci, karst spring	17.679228	43.016968	Freshwater	-	0	-
23	Čekrk, karst spring	17.673341	43.019067	Freshwater	+	0.89	34.69 ± 1.15
24	Bijeli Vir, main	17.656233	43.009189	Freshwater	+	0.44	34.58 ± 0.82

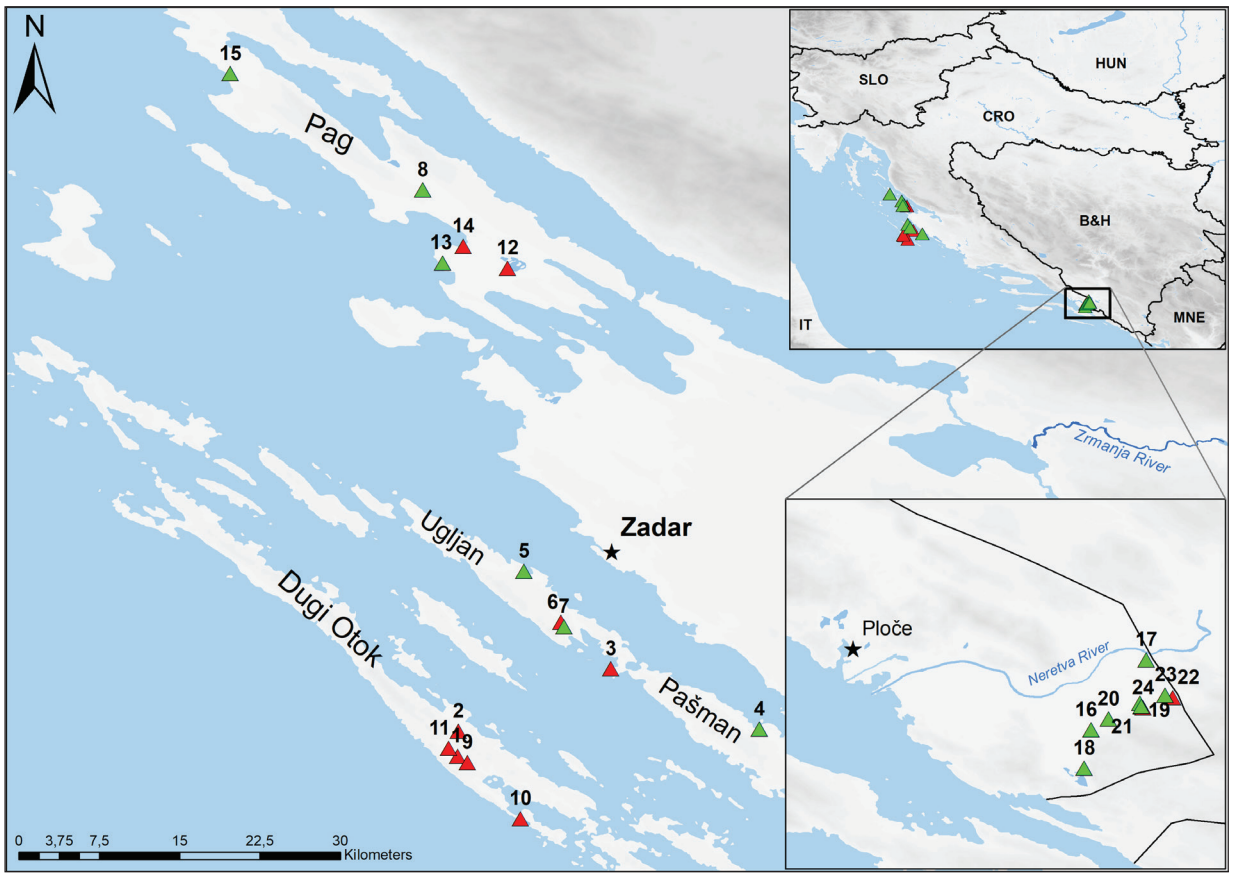

Fig. 2: Localities included in this study. Red triangles represent localities with no detection of *A. anguilla*, green triangles represent localities where *A. anguilla* was detected.

(Rocker Lafil300 OilFree Pump), as well as a 1L filtering unit (Nalgene™) and nitrocellulose filters (Sartorius® 47 mm diameter and 0.45 µm size pore) (Lawson Handley *et al*., 2019). Surface water samples were sampled in three different ways, depending on the size of the water body: i) for small water bodies sampling was performed from the bank, ii) for medium water bodies it was done by entering the water body using plastic waders, and iii) for large water bodies it was done by using a small rubber boat. Between every sampling site, all equipment was disinfected using 10% bleach and 96% ethanol. Samples were collected using a decontaminated plastic bottle, thoroughly rinsed by submerging them into the water just before the effective sampling, and wearing non-powdered gloves. Following the filtration process, the filter was removed and folded in quarters into a 1.5 mL tube using sterile forceps. The filters were stored in 1 mL of 100% molecular-grade ethanol, in a cooler box, until it was returned to the laboratory. Three independent natural replicates, each consisting of 1L of filtered water, were collected at each sampling location.

DNA extraction

DNA extraction from tissue

Muscle tissue from a live specimen caught in the Neretva River, Croatia, was used for genomic *A. anguilla* DNA extraction, which was done following the manufacturer's guidelines in the Qiagen DNeasy® Blood & Tissue Kit.

Environmental DNA extraction

To avoid contamination, extractions were performed in a separate sterile laboratory, different from the one in which the preparation of the qPCR mixture took place. Half of each filter was cut with sterilized tweezers and scissors, and dried for thirty minutes permitting the ethanol to evaporate from the filter. After this procedure, one half of the filter was further cut into small pieces and placed in a new 2 mL Eppendorf tube. The Qiagen DNeasy® Blood & Tissue Kit for DNA extraction was used, following the manufacturer's guidelines with slight modifications as shown in Baudry *et al.* (2021). The modifications were as follows, 450 µL of ATL buffer and 50 µL of Proteinase K were added to the fragment filter tube, vortexed for 15 seconds, and incubated at 56°C overnight. Then, 500 µL of AL buffer and 500 µL of 100% ethanol were added. The remaining steps were performed following the manufacturer's protocol. The extracted DNA was stored at -20°C until further analysis.

qPCR primers and probe specificity

Species-specific primers and probes used in this study were those developed by Weldon *et al.* (2020), tar-

geting a cytochrome *b* region of *A. anguilla* (Forward: Aangcytb1F 5'- TTGCCCTATTCTACCCGAACC-3', Reverse: Aangcytb1R 5'- ACAAGGCTAATACCCCGCC-3' and specific-fluorescent labelled probe: Aangcytb1P 5'- TTGGAGACCCAGACAACTTCACCCCGGCA-3'). The specificity of the primers was determined *in silico* using the primer-BLAST tool.

In vitro tests have already been carried out by Weldon *et al.* (2020) on 17 species, and they indicate the absence of amplification in these taxa (Table 2), attesting to the specificity of the primers for *A. anguilla*. Due to different biotic contexts in the Croatian Adriatic, especially in terms of faunal composition, we performed *in vitro* tests with 5 new species found in Croatian freshwaters, to test the specificity of the primers (Table 2). The qPCR parameters (primer and probe concentrations, and annealing temperature) used are those described and optimized in Weldon *et al.* (2020), providing good amplification yields.

qPCR treatments

For the detection of *A. anguila* DNA, we performed real-time PCR using the primers Aangcytb1F (5′–TTGCCCTATTCTACCCGAACC–3′) and Aangcytb1R (5′– ACAAGGCTAATACCCCGCC–3′), and a fluorescently labeled probe Aangcytb1P (5′–FAM–TTGGAGACCCAGACAACTTCACCCCGGCA–BHQ1–3′), designed by Weldon *et al*. (2020). All oligonucleotides were manufactured by Macrogen Europe. Each natural replicate (i.e., water sample) was analyzed in three technical/qPCR replicates, providing nine replicates per sampling station. Each qPCR reaction contained 10 µl of GoTaq® Probe qPCR Master Mix (Promega, USA), 0.2 µM of each primer, a 0.1 µM probe, 5 µl of DNA, and water up to 20 µl. Thermocycling and detection was performed on a qTower^3 (Analytik Jena, Germany), with the following protocol: denaturation at 95°C for 2 min, followed by 40 cycles of 95°C for 15 s and 59 °C for 1 min. Each assay included three replicates of the positive control – *A. anguilla* genomic DNA (extracted from *A. anguilla* muscle tissue as described above) and three replicates of 10x diluted positive control. Three replicates of the negative control (H_2O) were also included in each assay. Field positive controls weren't performed, since the sampling referred to past known presence data. Field negative controls were not performed as well, since the sampling protocol was based on published methodologies (e.g., Baudry *et al.,* 2021; Dubreuil *et al.,* 2022), showing the effectiveness of the disinfection protocol used in this study.

Positive signals were considered when a C_t value (cycle threshold; the value defining positive and negative amplifications) below 36 at a site was defined as "harboring *A. anguilla*", as well as if at least one replicate of the nine (per station) was positive (following Weldon *et al*., 2020). This threshold for positive results is validated and used in many published studies (Bedwell & Goldberd, 2020). It can be assumed that this amount of eDNA is low, however, we have taken a more cautious reading of our results. Most studies consider a positive qPCR result for a Ct value of <45 (e.g., Bedwell & Goldberg, 2020) or <42 (Agersnap *et al.,* 2017), but we have chosen to lower the limit to Ct <36 in this study, thus attesting to the robustness of our results.

Limit of detection (LOD) and limit of quantification (LOQ)

LOD and LOQ were calculated in accordance with the Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) guidelines (Bustin *et al.,* 2009). For that purpose, standard dilutions (of known concentrations) were done from an *A. anguilla* DNA extract (53,58 ng μL^{-1}, measured using Nanodrop Spectrophotometer), which were then treated in 10 replicates by

Table 2. List of fish species used to test primer specificity.

Species	Source	qPCR results
A. anguilla	this study	+
A. anguilla	Weldon *et al.* (2020)	+
Alburnus alburnus	this study	-
Rutilus rutilus	this study	-
Phoxinus lumaireul	this study	-
Squalius illyricus	this study	-
Salmo faroides	this study	-
Scardinius erythrophthalmus	Weldon *et al.* (2020)	-
Petromyzon marinus	Weldon *et al*. (2020)	-
Perca fluviatilis	Weldon *et al.* (2020)	-
A. rostrata	Weldon *et al.* (2020)	+ (weak)

qPCR. LOD corresponds to the lowest concentration at which organismal DNA can be detected by qPCR and LOQ corresponds to the lowest concentration at which targeted DNA can be quantified. Our qPCR results were modeled following Klymus *et al.* (2019), with slight modifications, as our concentrations were measured in ng μL^{-1}. The model was performed with the "*Best*" parameter for *LOD.FCT* and *LOQ.FCT* functions, and 0.7 for the *LOQ.threshold* function.

Data analysis

In this paper, all older literature data available was analyzed, together with recent data gathered by the author and colleagues, in the period from 2010 to 2021. All field data (sites, coordinates, and habitat) and lab results (qPCR positive replicates and mean Ct values ± SD) were compiled in Table 2. All data was further analyzed in QGIS v. 3.26 software (QGIS Development Team, 2022) to plot maps, both for the historic records of *A. anguilla* in Croatia and for detection based on the eDNA method from the current study.

Rstudio V1.1.463 (Core Team R Development, 2019) was used to perform statistical analyses and modelling. The Shapiro-Wilk normality test and the Bartlett homogeneity test were used to verify the normal and homogeneous distribution of the data ($p > 0.05$). Lastly, the effect of habitat type on detection efficiency and sensitivity was investigated with a one-way ANOVA.

Results

qPCR assays

In silico tests performed using the alignment primer-BLAST tool showed no risk of cross-amplification with non-target species and other closely related Anguillid species. Primers and the probe experiment set showed 100% specificity for *A. anguilla* and the best hit for another species is 96% specificity for *Crenicichla lepidota*, a cichlid species native to South America and absent from the Adriatic and the Mediterranean Sea.

In vitro testing confirmed this high specificity, with no DNA amplification of co-occurring species in Croatia (Table 1). Only a weak amplification for the American eel (*A. rostrata*) (Weldon *et al.*, 2020) occurred, but it does not pose a problem for the specificity of the tested primers and the probe, since this species, like the aforementioned *C. lepidota*, is *a priori* absent in Europe.

The qPCR assays show a high sensitivity, with LOD and LOQ corresponding respectively to concentrations of 5.1 $x10^{-4}$ ng.μL^{-1} and 8.1 $x10^{-4}$ ng.μL^{-1} (p-value < 0.05).

In-situ detection and habitat effect

The European eel was detected by the environmental DNA-based method in 13 out of the 24 studied localities, with an amplification rate ranging from four to nine, out of the nine technical replicates (Table 2, Fig. 2). qPCR sensitivity (mean Ct values) for these positive stations ranged from 28.9 ± 0.54, with a proportion of qPCR positive replicates reaching 100% (for Pag, Sega lagoon; Marine habitat), to 35.32 ± 0.76, with a proportion of qPCR positive replicates reaching 67% (for Ugljan, Vela Lamjana; Marine habitat). Interestingly, *A. anguilla* was detected in six localities at three Adriatic islands: Pag, Pašman, and Ugljan, but not at Dugi otok Island. Eel DNA was detected in seven out of the nine sampled localities in the Neretva River Delta (Table 2, Fig. 2).

Interestingly, the qPCR results show that *A. anguilla* can be detected in all prospected habitats, i.e., fresh water, marine water, and brackish water (Table 2). Eel eDNA was detected at two out of the four brackish sampling sites, seven out of the 13 freshwater sampling sites, and four out of the seven marine sampling sites.

Ratios of positive qPCR replicates reach 0.5 ± 0.58, 0.45 ± 0.46, and 0.48 ± 0.47 for brackish, freshwater, and marine habitats, respectively. No significant difference was found in this detection probability, but the sensitivity difference was significant (mean Ct values). The eel eDNA seemed to be significantly more frequently detected in marine and brackish habitats (32.11 ± 2.67 Ct and 31.9 ± 1.47 Ct) than in freshwater (33.6 ± 1.57 Ct) ($F = 7.67$; p-value < 0.001) (Fig. 3).

Discussion

Environmental DNA application

The use of eDNA as a tool for monitoring *A. anguilla* presence in Croatia was found to be accurate and reliable. We used the eDNA identification setup (primers and probe) developed by Weldon *et al*. (2020) to detect *A. anguilla* in Ireland. In the latter study, Weldon *et al*. (2020) have proven the specificity of the primers and the probe for the *A. anguilla* species through *in silico* and *in vitro* testing on co-occurring species, without any match possibility with them. In our study, we performed additional tests to prove the specificity of the primers and the probe for applications in Croatia, where other species inhabit naturally. *In silico* and *in vitro* tests showed that the qPCR assay developed by Weldon *et al*. (2020) for the detection of *A. anguilla* is species-specific enough to be also applied in the Croatian Adriatic, and LOD calculations show a high sensitivity of the method.

At all sites, sampling was conducted on surface waters. Although Burgoa Cardás *et al*. (2020) detected higher proportion of positive DNA amplifications in the bottom than in the surface water samples, other studies showed no difference between surface and subsurface water, even for benthic species (Hinlo *et al*., 2017; Forsström & Vasemägi, 2016). Furthermore, Weldon *et al*. (2020) also sampled surface water for the detection of *A. anguilla* and confirmed this method to be effective. The results of this study show that *A. anguilla* eDNA can be successfully recovered and amplified from freshwater, brackish, and

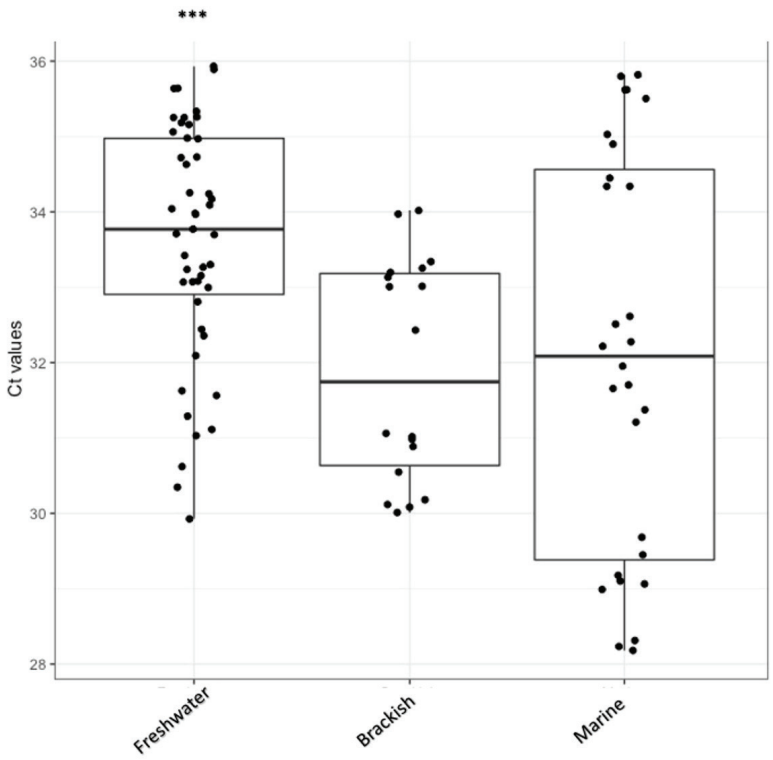

Fig. 3: Influence of habitat types, brackish, freshwater, and marine, on detection sensitivity (Ct values). The annotations (***) highlight a significant difference between the groups.

marine habitats. The qPCR results seem to highlight an effect of the habitat on detection by eDNA, especially on detection sensitivity (Mean Ct value). It seems that detection is less effective in freshwater than in brackish and marine habitats. However, this can be a consequence of the eel's ecological preferences, the sampling sites, as well as the characteristics of environmental DNA. First, the eel is a catadromous species, adapted to both freshwater and marine water, and therefore, its presence can be detected in the entire basin, from upstream to downstream reaches of rivers and their estuaries. This makes the sampling area very large and, depending on seasonal variations, the species may be absent from certain localities within the sampling systems (*e.g.*, upstream/freshwater). Second, the sampling conducted here represents nearly twice as many sites sampled in fresh water as those in brackish or marine water. This inevitably impacts the detection efficiency of the species, due to prospecting areas where the eel has never been detected, and therefore the probability of detection will be reduced in freshwater areas. Finally, eDNA is well-known to persist in the environment and diffuse downstream, sometimes up to several tens of kilometers (Deiner & Altermatt, 2014). In this case, eDNA will eventually end up in the downstream zones (brackish or estuarine/marine), well known to be the final recipient of the entire hydrographic flow. Nevertheless, the sampling was carried out in a thoughtful way, with points distributed from the upstream (freshwater) to the transition zones (brackish), then marine, thus giving precise and robust location data for *A. anguilla*. Thus, our study represents the only application of eDNA for research and monitoring purposes in Croatia, after the study on the olm, *Proteus anguinus* (Vörös *et al*., 2017). It is the first eDNA research of any fish species in Croatia, the first eDNA monitoring of *A. anguilla* in the Adriatic Sea and, to our knowledge, the first one in the Mediterranean region. Burgoa Cardás *et al*. (2020) applied eDNA analysis for monitoring the European eel in Spain, however, monitoring was performed in the rivers of the Atlantic drainage basin. Additionally, our study is the first one investigating the presence of the European eel in the Adriatic basin, a species of major ecological importance for which many conservation plans have been deployed around the world.

Detected eel populations

Conventional research methods using electrofishing did not prove to be very effective for detecting eels in the coastal lagoon habitats of the Adriatic Islands, since these are unique habitats with very high salinity, which makes electrofishing either inefficient or impossible. On the other hand, even in freshwater systems, electrofishing has certain limitations that affect its efficiency due to electrical conductivity, turbidity, habitat complexity, fish size, and species (Lieschke *et al*., 2019). Additionally, efficiency of electrofishing decreases with the increase in the width and depth of the stream, causing considerable variation in effectiveness, especially in large lowland systems (Pottier *et al*., 2020; Lyon *et al*., 2014). Using eDNA as an alternative to electrofishing sampling, even for freshwater fish communities; it is already established to be more effective than the traditional methods (McColl-Gausden *et al*., 2021). In some localities in this research (*e.g.*, 12 and 15 in Table 1), the surface layer of the water body is freshwater (approx. 50 cm), but below that is salt water, where electrofishing is inefficient. Eels

are usually hidden in the bottom layer, under rocks and vegetation, or buried in the mud (Froese & Pauly, 2021). Hand nets are ineffective during the day when the eels are hiding, and even if spotted they are very difficult to catch. Light traps could also be used, but some problems still remain, similar to hand nets and pull nets. De Graaf *et al*. (2010) discuss that the optimal positioning of light traps is still unknown, and their biggest problem is zero catches, demonstrating that light traps are not a suitable alternative. In addition to all this, it is necessary to consider that eel populations in these locations are expected to be smaller, since these are not their most optimal habitats, like river estuaries and lakes (Moriarty, 2003). However, ideal habitats appear to be strongly linked to areas with extensive freshwater-saltwater mixing. Some modest differences in eel detection among sample sites might be explained by the migratory behavior of eels and their environmental preferences. When glass eels arrive from the Sargasso Sea, they spend more time near a river's mouth than in the estuary (Harrison *et al*., 2014). Due to the mixing of saltwater and freshwater (brackish waters) in some Adriatic islands (especially Pag, Pašman, and Ugljan), the presence of the eel might be explained by this environmental condition. Furthermore, the detection of the European eel on these islands and its lack of detection on the Dugi Otok Island can be explained by the geography of these locations. The islands Pag, Pašman, and Ugljan are all positioned closer to the mainland and close to the Zrmanja River estuary, making them the most convenient locations for eels to migrate. The eel development period in continental waters ends with the silvering process, after which *A. anguilla* begins migrating to marine waters. Migrating silver-stage *A. anguilla*, like other diadromous fish, pass through fertile estuarine ecosystems with vast populations of birds, mammals, and other fish predators. Predation pressure on migratory fish in such areas may be considerable and it is known that cormorants (*Phalacrocorax* sp.), a frequent bird species in estuary habitats, prey substantially on smaller *A. anguilla* specimens (Keller, 1995; Barry *et al*., 2016). Sites 6 and 7 on Ugljan Island, although geographically close, are physically separated from one another. Site 6 is a pond divided from the sea, and it is connected to it only during high sea water (storm tides, low pressure atmospheric systems, etc.). So, this result indicates that there is no underground connection either, since the eels were not detected. On the other hand, Site 7 is a lagoon directly connected to the open sea waters, which makes it an easily accessible and a suitable locality for the eels. Therefore, smaller island habitats, such as aforementioned coastal lagoons and lakes, are suitable for eels escaping predatory pressure. Furthermore, it seems that *A. anguilla* is not present in habitats which are under heavy anthropogenic pressure. Site 3 on Pašman Island is positioned in the area of frequent ferry and catamaran lines (Zadar-Zaglav, Zadar-Sali, Zadar-Bršanj-Mala Rava, Zadar-Rava, etc.) with beaches attractive to tourists, and the eels were not detected there, indicating that heavy anthropogenic pressure makes this habitat not suitable for the eels.

On the other hand, *A. anguilla* is a common species in the Neretva River Delta and eel DNA detection is not surprising. The detection of eel DNA in the upper parts of this area, and outside of the main course of the river, is unsurprising as well, since it is known that eels migrate far upstream in the Neretva River, up to 100 kilometers inland, *e.g.*, to Hutovo Blato, Bosnia and Herzegovina (Has-Schön *et al*., 2008; Đađibegović *et al*., 2012). Burgoa Cardás *et al*. (2020) detected eels in the upstream parts of rivers during different sampling seasons (November, February, April, July). However, in the lower parts of rivers, eels were not detected in the November sampling. According to this, we detected *A. anguilla* at almost all sampling sites in the Neretva River (7 out of 9), positioned upstream from the river mouth. Furthermore, sampling for this research was performed during early December, which is the period of the early entry season of the glass eel (Burgoa Cardás *et al*., 2020).

Additionally, eel DNA detection in the karst springs (localities 18 and 21 in Table 1) was also expected, since *A. anguilla* uses underground corridors in its upstream migration (Dojmi, 1939). This is the reason why it can be found in karst poljes that have no direct surface water connection to the Adriatic Sea, such as Imotsko, Mostarsko, Popovo, and Dabarsko polje, positioned 150, 230, 300, and 550 meters above sea level (m.a.s.l.), respectively. (Ćurčić, 1916; Dojmi, 1939). It is also known that the eels migrate up to 100 kilometers upstream in the Neretva River, with maximum gained elevation of 640 m.a.s.l. (Ćurčić, 1916). Interestingly, sites 22 and 23 are geographically close karstic springs, but the eel has been detected in only one of them. The main problem with underground karstic water is that we usually cannot determine where the water comes from. Even though springs, sinkholes, or estavelles can be geographically really close, often they do not share the same water, due to different underground connections from those on the ground itself (Bonacci, 1999; Bonacci, 2015; Bonacci *et al*., 2013; Bonacci & Andrić, 2008; Palandačić *et al*., 2012).

Remarks on conservation

The European eel is an important species worldwide, both in fisheries and in aquaculture (Violi *et al*., 2015). This critically endangered species faces imminent and drastic population decline, with its current population at its all-time low (Pike *et al*., 2020). Management plans for the eels are being developed in order to protect and restore their populations through the reduction in anthropogenic mortalities and by enabling a high probability of escapement to the sea. Even though the implementation of management measures has shown certain improvement, the impact of those measures is still not adequate and the European eel remains a critically endangered species included in the last IUCN assessment (Pike *et al*., 2020). Another main problem is the limited understanding of the complex relationship between recruitment, the growth phase, and the escapement of eels, due to the lack of data on ecology and distribution of the species, especially in its southern range of distribution. Filling these

gaps will make management plans more efficient and allow a more comprehensive assessment of this species (Pike *et al*., 2020). Adriatic Sea harbors a large part of the *A. anguilla* stock, as it can be found in all Adriatic rivers. Furthermore, the presence of eels in bodies of water across the Croatian Adriatic islands highlights the importance of these habitats for its migrating silver-stage. These habitats can play an important role for the migration paths of the species and as a refuge from predators. In order to implement the Eel Regulation Act and design efficient Ecological Management Plans (EMPs), considerably more data is needed, especially on the distribution of this species. Once more, this study has demonstrated the efficiency of eDNA as a powerful tool in detecting rare and elusive species like *A. anguilla*. Similar to previous studies (Hänfling *et al*., 2016; Weldon *et al*., 2020; Burgoa Cardás *et al*., 2020), our study also suggests that this strategy might be a long-term solution for the detection and monitoring of anguillid species in their natural habitats. The application of eDNA provides the opportunity to asses populations and habitats which are important for European eels in the Adriatic Sea, to act fast in order to protect them, and to enhance eel stock recruitment in the long run.

Finally, it is necessary to emphasize the importance of this method due to its application in karstic waters. Eels use underground pathways regularly to enter and travel far inland (Ćurčić, 1916; Dojmi, 1939). This is also confirmed by the findings of eel DNA in Gacko polje (Croatia) (Fig. 1), where the only connection with the Adriatic Sea is through several sinkholes and underground waterways below the Velebit mountains. Similar situations can be observed in other karst poljes in Croatia, such as Ličko, Vrgoračko, Imotsko, and Sinjsko. As a result, the eDNA approach is also critical for detecting this species in karstic waters and for determining significant inland habitats and migration pathways.

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Appendix A. Historic data on *A. anguilla* presence in Croatia in the 10x10 km EEA grid.

0	10kmE460N247	4600000	2470000
1	10kmE460N248	4600000	2480000
2	10kmE461N247	4610000	2470000
3	10kmE462N246	4620000	2460000
4	10kmE462N247	4620000	2470000
5	10kmE462N248	4620000	2480000
6	10kmE463N245	4630000	2450000
7	10kmE463N246	4630000	2460000
8	10kmE464N246	4640000	2460000
9	10kmE466N242	4660000	2420000
10	10kmE466N248	4660000	2480000
11	10kmE467N247	4670000	2470000
12	10kmE468N245	4680000	2450000
13	10kmE468N246	4680000	2460000
14	10kmE469N245	4690000	2450000
15	10kmE469N246	4690000	2460000
16	10kmE472N237	4720000	2370000
17	10kmE472N243	4720000	2430000
18	10kmE473N237	4730000	2370000
19	10kmE473N241	4730000	2410000
20	10kmE473N242	4730000	2420000
21	10kmE473N243	4730000	2430000
22	10kmE474N234	4740000	2340000
23	10kmE474N235	4740000	2350000
24	10kmE474N240	4740000	2400000
25	10kmE476N232	4760000	2320000
26	10kmE476N233	4760000	2330000
27	10kmE476N235	4760000	2350000
28	10kmE476N236	4760000	2360000
29	10kmE477N232	4770000	2320000
30	10kmE477N235	4770000	2350000
31	10kmE477N236	4770000	2360000
32	10kmE478N236	4780000	2360000
33	10kmE479N231	4790000	2310000
34	10kmE479N232	4790000	2320000
35	10kmE479N235	4790000	2350000
36	10kmE479N236	4790000	2360000
37	10kmE480N230	4800000	2300000
38	10kmE480N231	4800000	2310000
39	10kmE480N232	4800000	2320000
40	10kmE480N233	4800000	2330000
41	10kmE482N228	4820000	2280000
42	10kmE482N229	4820000	2290000
43	10kmE483N229	4830000	2290000
44	10kmE484N229	4840000	2290000
45	10kmE484N232	4840000	2320000
46	10kmE485N229	4850000	2290000
47	10kmE486N228	4860000	2280000
48	10kmE486N229	4860000	2290000
49	10kmE486N230	4860000	2300000
50	10kmE487N228	4870000	2280000
51	10kmE489N228	4890000	2280000
52	10kmE490N228	4900000	2280000
53	10kmE492N224	4920000	2240000
54	10kmE492N225	4920000	2250000
55	10kmE493N221	4930000	2210000
56	10kmE493N224	4930000	2240000
57	10kmE494N223	4940000	2230000
58	10kmE494N224	4940000	2240000
59	10kmE494N225	4940000	2250000
60	10kmE498N220	4980000	2200000
61	10kmE498N221	4980000	2210000
62	10kmE499N220	4990000	2200000
63	10kmE500N219	5000000	2190000
64	10kmE501N219	5010000	2190000