ORIGINAL ARTICLE

New boundaries: Redefining the geographical range of a threatened fish through environmental DNA survey

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Abstract

Accurate data on the distribution and population status of threatened fish species are fundamental for effective conservation planning and management. In this work, in order to reassess the distribution of the globally threatened Evia barbel, *Barbus euboicus*, we undertook an environmental DNA (eDNA) survey coupled with conventional electrofishing, focusing on major river basins in Evia Island in proximity to its known occurrence in a single Evian basin (Manikiatiko stream). For comparison purposes, we conducted eDNA sampling in several locations in the geographically closest continental river basin, the Sperchios basin (Central Greece) which hosts the closely related *Barbus sperchiensis*. Our results expand the known range of the Evia barbel on Evia adding four new river basins, apart from its type locality (Manikiatiko stream (EV3)). In a single Evian River, where the species had never been located before, there was also a positive eDNA signal for *Barbus sperchiensis* within the same basin. The research confirms the occurrence of Evia barbel in a wider geographical area, highlighting however the sensitive conservation status of the species due to its still very narrow geographical distribution. The biogeographical implications of our study, as well as potential conservation interventions, are discussed.

KEYWORDS
eDNA, electrofishing, endangered species, freshwater fish, monitoring, river basin, spatial distribution

1 | INTRODUCTION

Globally, almost 30% of IUCN assessed Red Listed species are currently threatened with extinction (https://www.iucnredlist.org/); mainly due to anthropogenic habitat degradation and alien species impact, coupled with effects of climate change (Hooper et al., 2012; Jarić et al., 2019; Sala et al., 2000). Freshwater vertebrates are even more imperiled, with a twofold higher rate of decline compared to terrestrial or marine organisms (Dudgeon et al., 2006; McRae et al., 2017; Reid et al., 2019). Accurate knowledge of the presence and composition of threatened freshwater fish species populations is critical for the implementation of proper management strategies and for maximizing conservation success (Evans & Lamberti, 2018; Penaluna et al., 2021; Ushio et al., 2018).

Environmental DNA (eDNA), a molecular technique that relies on the detection of DNA traces from tissues of organisms in their environment, is currently at the forefront of aquatic conservation efforts as a less labor intensive, less invasive, more cost effective and more reliable monitoring method (especially for rare aquatic species), when compared to conventional fish sampling methods (Evans et al., 2017; Thomsen & Willerslev, 2015). Furthermore, eDNA-based surveys can permit the more reliable identification of target taxa compared with morphological identification that can be difficult in the case of closely related and "cryptic" species (Evans & Lamberti, 2018; Mauvisseau, Burian, et al., 2019). They can thus ultimately resolve uncertainties related to taxonomy and species distribution and often lead to revisions of the target species' range (Hobbs et al., 2020; Rees et al., 2014; Thomsen et al., 2012).

The freshwater Evia barbel *Barbus euboicus*, is a characteristic example of a fish species whose geographical distribution and population status remains unclear seventy years after its first description from a stream on Evia (Euboea) island (Manikiatiko stream, eastern-central Evia; Stephanidis, 1950). This relatively small rheophilic barbel is restricted to Evia, a continental island running parallel to the shores of Eastern Central Greece and hosting a species-poor native fish fauna (Zogaris & Economou, 2017). Apart from the Manikiatiko population, *Barbus* populations of northern and central of Evia have been variously assigned subsequently either to *B. euboicus* (Stephanidis, 1971; supported by a recent genetic study, Kyralová et al., 2019) or to the more widely distributed *Barbus sperchiensis* (Barbieri et al., 2015; Kottelat & Freyhof, 2007). The complicated biogeographical puzzle is due both to species misidentification (since solely morphological examination was applied in the past) and changing nomenclature that has affected several *Barbus* species in the Balkans (Economidis, 1989; Vavalidis et al., 2019).

All previous authors agree that the Evia barbel is seriously threatened (Freyhof et al., 2020). *Barbus euboicus* is listed as Critically Endangered (CR) in the IUCN Red List of Threatened Species and the Greek Red Data Book (Legakis & Maragou, 2009) and is a species of EU conservation concern listed in Annex II of the 92/43/EC Habitats Directive (Barbieri et al., 2015). Recently, it has been included as one of the five freshwater fish species that will be most impacted by climate change (Jarić et al., 2019). Thus, its genetic and geographical isolation, coupled with its susceptibility to projected climate change, render it one of the top priorities for species conservation in Greece and Europe (Barbieri et al., 2015).

The aim of the current study was to delineate the exact geographical range of *B. euboicus* in Evia Island and in central Greece comparatively with its congener, and the geographically closest barbel species, *B. sperchiensis* using aquatic eDNA detection, and to compare the efficacy of eDNA detection with abundance data obtained through standardized electrofishing. Based on our results, we discuss the biogeographical implications of our findings, prioritize *B. euboicus* habitats for conservation and discuss the prospects of promoting future translocation/reintroduction actions.

2 | MATERIALS AND METHODS

2.1 | Study area and sample collection

Field work was conducted in August 2019 and January 2022, using both conventional electrofishing and eDNA sampling, to obtain data on *Barbus* populations in six basins in Evia Island (sites EV1-8, Figure 1, Table 1) and in the Sperchios basin in Central Greece (sites SP1-7, Figure 1, Table 1).

2.2 | Environmental DNA and fish sampling

At each location, three independent (max 1 L) water samples were collected (Table 1) using a sterile polypropylene ladle and placed into a sterile plastic bag (Whirl-Pak® 1242 mL Stand-Up Bag Merck®). Samples from rivers consisted of pooling water subsamples regularly sampled from across the width of the rivers, by moving upstream, in order to avoid disturbing the sediments. Samples from each location were then filtered with a 50-mL syringe (sterile Luer-Lock™ BD Plastipak™) through a sterile 0.45 μm Sterivex™ HV filter (Sterivex™ filter unit, HV with luer-lock outlet, Merck®, Millipore®). Sterivex filters were then immediately fixed with 2 mL of absolute ethanol as Buffer and stored at room temperature (range 11–20°C) until the end of the fieldtrip Sterile equipment and disposable nitrile gloves were used during the sampling process and replaced at each location to avoid contamination. Samples were subsequently transported on dry ice to the United Kingdom (max. 8 days after collection, including journey time by air) and stored at −80°C prior to DNA extraction.

Following eDNA water sample collection, fish data were collected using standardized electrofishing used widely in the EU WFD procedure in Greece (method details provided in Zogaris et al., 2018). Electrofishing was conducted during daylight hours as the species are diurnal, with an EFKO electrofishing DC unit (Honda 7 kVA generator, 150 m cable, 1.5 m anode pole, 6 A DC output, voltage range 300–600 V). The sampling team consisted of three members, one operator of the anode, one netter collecting the stunned fish and one data recorder/operator of the deadman key. Briefly, a single electrofishing pass was conducted at a section of the target stream of approx. 100 m without using stop nets, since the fished section was usually demarcated by physical boundaries, such as riffles or small barriers, to minimize fish escape during electrofishing. In all cases, effort was made by the survey team to sample in a near-complete manner, that is, thoroughly sampling the entire river channel and covering all available instream habitats in order to get a representative sample of the fish community. The fished area was carefully estimated in order to calculate fish density values, expressed as number of fish caught/m^2 fished area. Captured fish were identified to species level (nomenclature following Barbieri et al., 2015), counted and then released into the water. Fin clips from the target species were obtained from individuals caught at their type localities
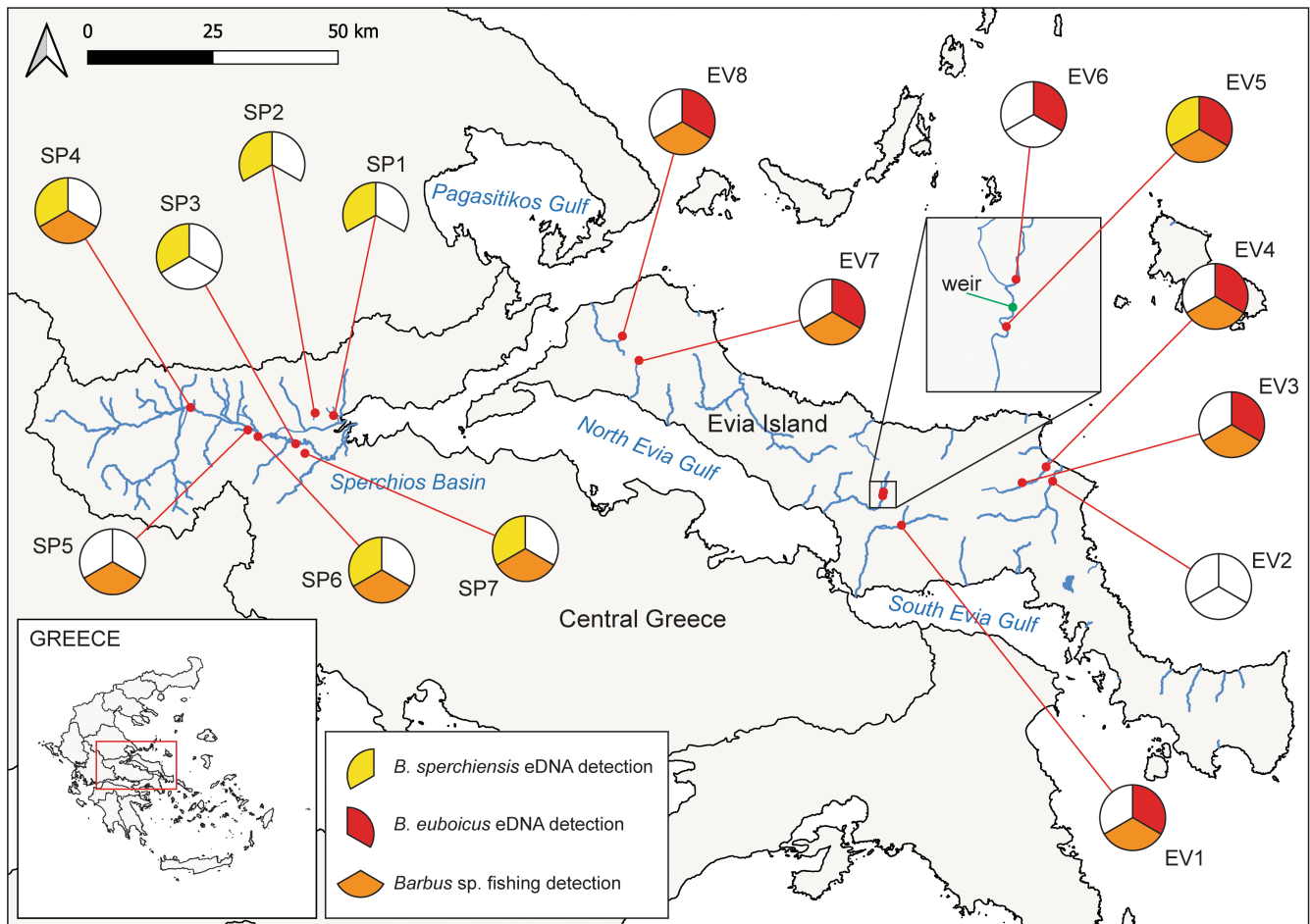
FIGURE 1 Sites sampled in Evia Island (six basins, sites EV1-8) and in Central Greece (Sperchios basin, sites SP1-7) targeting barbel populations (in 2019 and 2022), with eDNA results for *B. sperchiensis* and *B. euboicus*, and fishing results for *Barbus* sp. Red circles represent sampling sites, blue lines main water bodies in Evia and the Sperchios basin. Yellow pie charts represent eDNA detection of *B. sperchiensis*, red pie charts eDNA detection of *B. euboicus*, and orange pie charts barbel detection through electrofishing. In SP1 and 2, no fishing was performed due to high depth and/or salinity.

(*B. euboicus*: EV3; *B. sperchiensis*: SP4) and anesthetized with clove oil before being rereleased. Fin clips were also taken from the following sympatric species: *Gambusia holbrooki*; *Pseudorasbora parva*; *Squalius vardarensis*; *Pungitius hellenicus*; *Alburnoides econonoui*, *Gasterosteus gymnurus* and *Pelasgus marathonicus*. These were used for assay validation to check for species specificity.

At each sampling site, a series of physico-chemical and habitat parameters were recorded. Specifically, conductivity (μS/cm), salinity (ppt), dissolved oxygen (mg/L), pH, and water temperature (°C) were recorded in situ using a portable multiparameter Aquaprobe AP-200. Depth was recorded with a probe, while water flow was estimated visually, in a semi-quantitative way, using a six-class system (<0.1 m/s, 0.1–0.25 m/s, 0.25–0.5 m/s, 0.5–0.75 m/s, 0.75–1, >1 m/s). Other habitat characteristics determined were mean wetted width (m), shadedness (%), substrate coarseness, that is, ≥63 mm (%), riparian vegetation cover (%), and aquatic vegetation cover (%). Finally, the percentage of different habitat types sampled, that is, pool, glide, run, and pool, were also recorded for each study site.

2.3 | Environmental DNA analysis

Methods largely followed those described in Mauvisseau et al. (2020) with some adaptations. Species-specific primers and probes were designed using pre-existing sequences available via GenBank and targeting the cytochrome B gene (CytB) of *Barbus euboicus* (Stephanidis, 1950) and cytochrome C oxidase subunit 1 (COI) of *Barbus sperchiensis* (Stephanidis, 1950). The primers and probe were developed using the NCBI Primer-BLAST function (https://blast.ncbi.nlm.nih.gov/Blast.cgi; Ye et al., 2012) and the freely available online tool PrimerQuest™ (https://eu.idtdna.com/pages/tools/primerquest).

Assay specificity was tested in silico against DNA sequences retrieved from the NCBI database (National Centre for Biotechnology Information; https://www.ncbi.nlm.nih.gov/) from 29 fish species known to be and/or potentially present in the same ecosystems with the targeted organisms (see Table S1 in the supplementary information). The primers, probes and the sizes of the fragments amplified are provided in the supplementary information (Table S2) along with

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TABLE 1 Sampling sites and basins (in 2019 and 2022), fishing detection for barbel (densities, inds/m^2), eDNA detection (number of positive qPCR replicates for *B. sperchiensis* and *B. euboicus*.

Site	Basin	Region	Date	*Barbus* sp. (inds/m^2)	*B. sperchiensis* eDNA	*B. euboicus* eDNA	Volume (mL)/R	pH	Temp (°C)	Aq Veg
EV1	Lilas str.	Evia island	26/08/19	1.30	0/12	9/9 (3,3,3)	1000	8.04	23.50	25
EV2	Xondros str.	Evia island	26/08/19	0.00	0/12	0/9	1000	7.55	22.40	5
EV3	Manikiatiko str.	Evia island	27/08/19	AMB	0/12	6/9 (3,2,1)	1000	7.89	20.40	-
EV4		Evia island	27/08/19	0.19	0/12	4/9 (2,2,0)	1000	7.25	19.80	28
EV5	Messapios str.	Evia island	17/01/22	0.15	3/12 (2,1,0)	9/9 (3,3,3)	1000	8.14	12.40	0
EV6		Evia island	17/01/22	0.00	0/12	6/9 (3,2,1)	1000	7.88	12.31	15
EV7	Kokkinomilia str.	Evia island	28/08/19	0.09	0/12	5/9 (3,2,0)	1000	8.29	18.10	40
EV8	Istiaia str.	Evia island	28/08/19	AMB	0/12	8/9 (3,3,2)	1000	8.19	20.80	0
SP1	Sperchios R.	Central Greece	18/01/22	NP	9/12 (4,3,2)	0/9	300	7.85	8.38	0
SP2		Central Greece	18/01/22	NP	2/12 (1,1,0)	0/9	920	7.53	15.88	50
SP3		Central Greece	19/01/22	0.00	10/12 (4,4,2)	0/9	1000	7.95	7.97	30
SP4		Central Greece	29/08/19	0.23	12/12 (4,4,4)	0/9	1000	7.92	22.20	14
SP5		Central Greece	29/08/19	0.09	0/12	0/9	1000	7.85	17.17	75
SP6		Central Greece	18/01/22	0.40	6/12 (4,2,0)	0/9	850	7.90	13.11	0
SP7		Central Greece	18/01/22	0.01	3/12 (2,1,0)	0/9	1000[a]	7.87	11.81	24

Note: Value in brackets is the number of qPCR positives in each field replicate. Volume of water sampled for each field replicate (Volume (mL)/R), field pH, water temperature measurements, and coverage of aquatic vegetation are also provided for each location.

Abbreviations: AMB, non-quantitative fishing performed, confirming the presence of a barbel species; NP, no fishing performed.

[a]1000 + 1000 + 500 mL filtered.

details of their alignment with those non-target species later used for in vitro validation (Table S3). Following in silico validation, the specificity of each assay was tested in vitro with qPCR using DNA extracted from the following co-occurring species: *Alburnoides economoui*, *B. euboicus*, *B. sperchiensis*, *Gambusia holbrooki*, *Gasterosteus gymnurus*, *Pelasgus marathonicus*, and *Squalius vardarensis*. DNA was extracted from tissue samples of these species using the Qiagen DNeasy® Blood and Tissue Kit following the manufacturer's instructions.

eDNA was extracted from the filters with the Qiagen DNeasy® Blood and Tissue Kit, following the extraction workflow for Sterivex filters outlined in Spens et al. (2017). Extraction of eDNA samples was performed in a separate clean PCR-free room (different than that used for extraction of the tissue samples identified above).

Primer specificity was assessed using PCR before conducting qPCR. qPCR reactions were performed on an ABI StepOnePlus™ Real-Time PCR (Applied Biosystems). The specificity of each assay was further confirmed by qPCR using two replicates of DNA extracted from the different individuals of the species mentioned above. qPCR protocols and conditions were the same across all target species. These consisted of a 15 μL final volume, using 7.5 μL of qPCRBIO Probe Mix Hi-ROX (PCRBiosystems), 0.3 μL of each primer, 0.15 μL of probe, 4.75 μL of ddH2O, and 2 μL of extracted DNA. Concentrations of primer and probe were 10 nML^{-1}. The reactions were run on a fast presence/absence test using the following cycling parameters: 2 min denaturation at 95°C, followed by 45 cycling steps of 5 s at 95°C and 20 s at 61°C (after Weldon et al., 2020).

Each PCR plate included a prepared serial dilution of standard of genomic DNA, extracted from the target species' tissue using the protocol described earlier, in triplicate. The concentration of the standard was confirmed using a Qubit 4.0 Fluorometer (Thermo Fisher Scientific). The standards, typically seven per plate, provide a regression line from which the unknown quantities of the DNA extracts can be estimated. A positive result was recorded for each sample if amplification reached the Ct threshold in one or more of the PCR replicates. The dilution series ranged from 10^{-1} to 10^{-8} using ten replicates per plate per dilution step allowing for the assessment of the limit of detection (LOD) and limit of quantification (LOQ) as detailed in Klymus et al. (2020). All eDNA samples were analyzed using three field replicates, *B. sperchiensis* samples were each analyzed with four technical replicates (overall number of replicates = twelve per location). The number of technical replicates was decreased to three per field replication for *B. euboicus* due to good levels of inter-sample consistency within PCR replicates (overall number of replicates = nine per location). Each qPCR plate also contained three replicates of six dilution points ranging from 10^{-3} to 10^{-8} as positive control and three negative controls using DNA-free water in place of the sample.

2.4 | Statistical analysis

We used the RShiny app "eDNA 1.0" (Diana et al., 2021), which implements the Bayesian occupancy modeling framework introduced

by Griffin et al. (2020), to generate estimates of the probability of site occupancy (ψ) and detection during field sampling (θ) and laboratory testing (p), including false-positive and false-negative detections for both target species. This provides estimates of the probability of target species' DNA being present in samples from sites which are occupied (θ_{11}) and unoccupied (θ_{10}) as well as the probability of target species' DNA being present in a qPCR replicate when taken from sites which are occupied (p_{11}) and unoccupied (p_{10}). False negatives from the field and laboratory samples are given by $1-\theta_{11}$ and $1-p_{11}$ respectively. All other modeling settings were left as default values as recommended by the RShiny app creators (Diana et al., 2021).

Following removal of correlated covariates, pH, salinity, mean flow, wetted width, substrate coarseness, and aquatic vegetation cover were included as standardized covariates. There was not enough variation in the amount of water filtered at each site to provide meaningful comparisons and therefore this was not included in the analyses.

3 | RESULTS

In Evia Island, seven out of the eight sampling sites tested positive for *B. euboicus* with eDNA (Figure 1, Table 1). These sites belong to the basins of Lilas, Manikiatis, Messapios, Kokkinomilia (Rovies), and Istiaia (Xirias) stream. Through electrofishing, the presence of a barbel species was confirmed in six of these sites (in all the above basins), but visual species identification was not possible. In one stream in Evia (Mourteri str.) we confirmed the absence of a barbel species, with both electrofishing and the eDNA sampling. All sites in Evia Island, tested negative for *B. sperchiensis* with eDNA, with the exception of one site (EV5 in Messapios R.) that gave a very strong signal for *B. euboicus* but also a weaker signal for *B. sperchiensis* (Figure 1, Table 1).

In the Sperchios basin, six out of the seven sampling sites tested positive for *B. sperchiensis* with eDNA. In four out of those six sites that electrofishing was performed, we were able to confirm the presence of a barbel species also through fish sampling. All Sperchios sites tested negative for *B. euboicus* with eDNA (Figure 1, Table 1).

Occupancy modeling indicated that the probability of occurrence (ψ) was below the model's expected value of 0.5 for both species. The probability of true positives was high for both species within both field (θ_{11}) and laboratory (p_{11}) samples at or marginally below the expected probability of 0.9 (range 0.838–0.900). The probability of false positives was below the expected rate of 0.1 for both species in field (θ_{10}) and laboratory (p_{10}) samples (Table 2). Posterior conditional probabilities of species absence given x positive qPCR replicates $(1-\psi(x))$ indicate that two positive PCR replicates represent a probability of less than 25% represents a false positive for *B. euboicus*, whereas for *B. sperchiensis* three positive PCR replicates are required to provide an occupancy probability less than 25% (Figure 2). Therefore, positive records at sites where the number of PCR replicates does not reach this threshold may need to be treated with greater caution. All sites where *B. euboicus* was recorded reached threshold in at least one field sample. For *B. sperchiensis*, three sites (EV5, SP2, and SP7) do not reach the threshold.

4 | DISCUSSION

In the current study, the use of eDNA-based detection methods confirmed the occurrence of *B. euboicus* in three streams of northern and central Evia Island with previously recorded barbel populations (Lilas stream, Kokkinomilia stream, and Istiaia stream), as well as its occurrence in the previously unrecorded drainage of Messapios in central Evia island. In addition, the eDNA analysis clearly limited the occurrence of *B. euboicus* to the island of Evia, as the geographically closest barbel populations of the Sperchios river basin tested negative for *B. euboicus*. This indicated an advantage of eDNA-based detection methods for surveying cryptic species, since the two *Barbus* species are phenotypically very similar, save for minor idiosyncrasies in dorsal fin spine serration and scale counts (Kottelat & Freyhof, 2007) which may vary with age and size, as well as with habitat conditions.

The occupancy modeling approach we employed estimates occupancy, false-positive and false-negative errors (Griffin et al., 2020). The results suggested that our assay provided a low likelihood of

TABLE 2 Posterior summaries of the probabilities of occurrence and detection, including the probability of false positive and false negatives, at both the field and laboratory stages of the analyses.

Parameter		Expected	Barbus euboicus		Barbus sperchiensis	
			Posterior mean	95% posterior credible interval	Posterior mean	95% posterior credible interval
Occurrence (ψ)		0.5	0.352	0.092, 0.685	0.252	0.055, 0.600
Probability of true positives	Field (θ_{11})	0.9	0.900	0.677, 1.000	0.875	0.530, 0.988
	Lab (p_{11})	0.9	0.850	0.691, 0.966	0.838	0.480, 1.000
Probability of false negatives	Field (θ_{10})	0.1	0.061	0.007, 0.210	0.068	0.008, 0.216
	Lab (p_{10})	0.1	0.043	0.004, 0.190	0.047	0.009, 0.110
Probability of false positives	Field ($1-\theta_{11}$)		0.100		0.125	
	Lab ($1-p_{11}$)		0.150		0.162	

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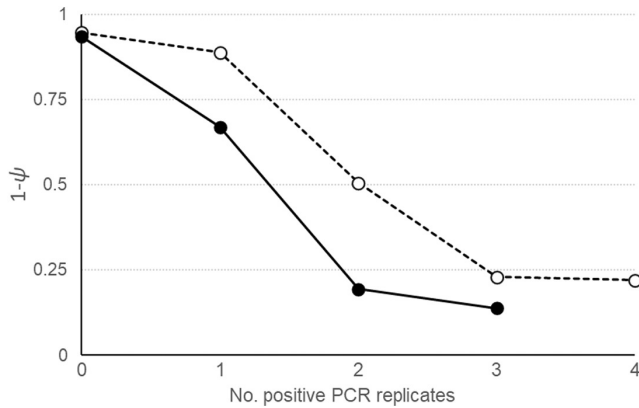Open Access

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FIGURE 2 Posterior conditional probability of species presence given x positive qPCR replicates for *B. euboicus* (black circles) and *B. sperchiensis* (open circles).

false positives in the field (θ_{10}) and lab (p_{10}) with values for both parameters being lower than the expected 10% for both species (Table 2). This suggests that most detections were likely to be true detections. However, the results for *B. sperchiensis* also indicate that two or fewer qPCR positives per individual field replicate represent greater than a 50% probability of this representing a false positive. Therefore, some of the sites at which *B. sperchiensis* was recorded solely by small number of qPCR positives, most notably EV5 and SP3 (Table 1), would benefit from further survey effort.

eDNA survey proved more efficient than electrofishing in precisely mapping the distribution of *B. euboicus*, as it detected the species at two locations on the main stem of Messapios River, at EV5 and the more upstream EV6, whereas conventional fish sampling failing to detect the species in the latter, possibly due to lower population densities (Penaluna et al., 2021) or because the electrofishing survey occurred downstream of the population loci (e.g. Shogren et al., 2017). The efficacy of the eDNA-based detection method was also exemplified in the Sperchios basin by the detection of *B. sperchiensis* in site SP3 where both our (and previous) fish sampling efforts have failed to detect the species. Conversely, shortcomings in the eDNA-based detection method were evident in the non-detection of *B. sperchiensis* in SP5 where the species was caught with electrofishing during our campaign. Failed detection using eDNA or "false negative" errors could be caused by a variety of factors including methodological errors in the collection, extraction or amplification of DNA or environmental factors such as the presence of a greater concentration of DNA inhibitors, hydrological drivers which may flush out eDNA or physiological factors which may have temporarily decreased the local input of eDNA to a concentration below the limit of detection (see Burian et al., 2021). It may be notable that, while no site covariates were found to have a significant impact on detection rates with the occupancy modeling analysis, the two sites with the highest coverage of aquatic vegetation (SP5: 75% and SP2: 50%) yielded very little or no target species' DNA (Table 1). Dense stands of aquatic vegetation have previously been posited as a barrier to eDNA dispersal and hence a factor in the presence of false negatives (e.g., Biggs et al., 2015), but its impacts have not,

to our knowledge, been systematically studied. It is possible that the time between samples being collected and stored in the −80°C freezer, allied to environmental temperature fluctuations, may have impacted the integrity of DNA within the samples, but there appears to be little evidence that the samples collected at the start of the fieldwork sessions contained less DNA that those collected at the end.

5 | BIOGEOGRAPHICAL IMPLICATIONS

There are still aspects of the distribution of barbels in Evia and central eastern Greece that require careful biogeographical research; the distributional data provided by the current survey introduce a few new questions. The double positive eDNA signal (for *B. euboicus* but also a weaker one for *B. sperchiensis*) at site EV5 in Messapios stream is unexpected. The most parsimonious explanation is that this is a false positive. The numbers of positive qPCR replicates within each field replicate are low and do not individually provide an occupancy probability greater than 50%. However, as more than one field replicate provided positive qPCR amplification (see Table 1), other explanations should be considered and the real presence of *B. sperchiensis* at this location not totally discounted. One alternative explanation could be that this section of the river hosts both a naturally occurring barbel population, as well as a translocated established population. Due to the currently defined distribution of *B. euboicus* one would consider the *B. sperchiensis* as the possible translocated species. However, this is not clearly evident by the geographical position of the host site and the paleogeography of the North Evia Gulf which provided the potential for past freshwater connections between Evia and Central Greece's eastern coast drainages. During the last glacial maximum, a former Pleistocene Lake existed in the mid-section of the Northern Evian Gulf (Sakellariou et al., 2007) and several rivers from both the mainland and the current Evia island presumably drained into this common water body. Since *B. sperchiensis* is the most widespread species on the opposite mainland (in both the Spercheios and Pagasitikos Gulfs of the Western Aegean Ecoregion), one may expect that this species would be widespread on Evia's west and north coastal streams. In fact, this was the former explanation which provided for the misidentification of several populations on Evia Island purporting that *B. euboicus* be isolated to the Manikiatis in the extreme eastern part of the island (Kottelat & Freyhof, 2007). There is no parsimonious path to the explanation of the current state of the two barbel species distributions on Evia, since at three points across the North Evia Gulf, *B. euboicus* dominates in Evia, that is, at Istiaia across from the Pagasiticus Gulf entrance, at Messapios and Kokkionomilia across from the Sperchios and other rivers of Eastern Central Greece. Interestingly the Lilas river, which also hosts *B. euboicus*, has its current estuary in the South Evia Gulf, a region which has no other barbel populations in any other biogeographically related streams (Economou et al., 2007). As in the North Evia Gulf, former drainages along the gulf, which are now isolated, were connected and fish populations could merge

even during the beginning of the Holocene, less than 10,000 years ago (Lykousis, 2009; Perissoratis & Conispoliatis, 2003). No other native *Barbus* sp. exist anywhere in the southern part of the Western Aegean ecoregion (Barbieri et al., 2015).

According to the currently accepted phylogeny *B. euboicus* is closely related to *B. peloponnesius* (Geiger et al., 2014), a species that has its distribution mainly west of the Pindos cordillera, in the Peloponnese and western Greece. No populations of *B. peloponnesius* exist in the Western Aegean Ecoregion. Two general hypotheses to explain the *Barbus* species distribution on Evia may be proposed: (a) either the current distributions of the barbels are a product of human translocations to a greater or lesser degree; and/or, (b) they are a product of a high extinction rate and consequentially define several isolated refugia. The rivers of Evia are of special interest because long-term spring-fed perennial streams exist, and these are known to host several endemic aquatic invertebrates (Zogaris & Economou, 2017). The distributional data of the barbels on the island is still incomplete and more detailed genetic work is required to help answer and interpret the questions posed here. In taking a precautionary approach toward biogenetic conservation we feel we should currently define all of the island's *Barbus* populations as "natural" and consider *Barbus euboicus* as a conservation priority; further genetic and biogeographic research should help clear the picture in the near future.

6 | CONSERVATION OPTIONS

Barbels in Evia Island are found along rather short stretches of streams. The stream flow is often interrupted by intermittent or ephemerally flowing sections (e.g., over karstic limestone substrate), by natural waterfalls and artificial barriers. Some river sections have very low barbel population densities; documented from our observation at lower and mid sections of the Istiaia and Kokkinomilia streams. It is, however, not unusual, that although there are adequate and suitable fish habitat areas in fairly large stream systems, these do not currently host barbels (e.g., Kireas in Northern Evia and spring-fed streams in Southern Evia) (Economou et al., 2007; and recent HCMR surveys). All extant Evia barbel populations inhabit streams that have human-induced water stress with extensive areas of varied flow regimes, with water abstraction and artificial barriers to fish movement constituting pressures on surviving populations and the main threats to the species (Barbieri et al., 2015). Thus, specific water management plans at the catchment scale should be drafted for these mixed perennial-intermittent aquatic systems, fulfilling both the minimal environmental flow (e-flow) requirements of the species, as well as human water needs (Arthington et al., 2018; Peñas et al., 2014). As in many parts of Greece, small artificial barriers such as road fords, weirs, and bridge foundations may present chronic dispersal challenges for fish to move upstream or into tributaries which may locally maintain adequate instream habitats for the species. Although these small streams may not be threatened by large-scale water development, such as hydropower (Freyhof et al., 2020), they are threatened

by further water abstraction works (for irrigation and potable water) and the effects of multiple stressors such as combined climatic, meteorological, and direct localized human-induced pressures (such as increasing longitudinal fragmentation by barriers). Because of the longitudinal fragmentation and poorly charted artificial barrier problems (Panagiotou et al., 2021) it is not possible to apply species distribution modeling or other spatial statistics tools to ascertain or explore the localized distribution of each population per drainage basin. The exact distributional and population data for each drainage is an imperative for conservation planning.

Wide-ranging exploratory inventory, mapping and field monitoring of the water bodies hosting extant *B. euboicus* populations should be conducted, both by conventional fish sampling methods and eDNA, to delineate the exact upper and lower reach limits of their habitats and identify "hotspots" for their conservation (Hobbs et al., 2020). The hotspot and important areas inventory and assessment will assist in the designation of new protected areas or support special care where water management and other water-centered development actions are being planned. A complete mapping of barbel distribution will assist plans to restore longitudinal connectivity, since many areas have small artificial barriers (Panagiotou et al., 2021).

Given the limited number of the stream systems currently hosting *B. euboicus*, an obvious conservation strategy is the translocation of the species in other aquatic systems within the now extended geographical range of the species in Evia Island, based on the current study. However, prior to any translocation trial, a genetic variation study at the population level should be conducted in order to identify *B. euboicus* populations that are potentially in separate evolutionary trajectories and thus should be treated as distinct conservation units (CUs, Mamuris et al., 2005; Vogiatzi et al., 2014). A conservation translocation also requires a detailed feasibility assessment prior to implementation, to indicate the potential release water bodies that fulfill several criteria related to several physical, environmental, and biotic factors, issues related to the ecology and life history of *B. euboicus*, as well as administrative, economic, and sociopolitical issues (Kalogianni et al., 2023) It also requires the implementation of the translocation according to the strict IUCN guidelines, following population viability analysis to define optimal stocking density and translocation frequency, as well as rigorous post-release monitoring of the release water bodies with standardized methods to assess the success of the translocation action (Berger-Tal et al., 2020; Cochran-Biederman et al., 2015; Fischer & Lindenmayer, 2000; George et al., 2009; Kalogianni et al., 2023). Due to the projected effects of climate change flow variations (Jarić et al., 2019) and the catastrophic impacts of possible extreme drought phenomena, we urge translocation feasibility studies for *Barbus euboicus* in Evia Island.

AUTHOR CONTRIBUTIONS

CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest.

DATA AVAILABILITY STATEMENT

Due to the potentially sensitive nature of the specific species locality data, data that support the findings of this study are available from the corresponding author upon reasonable request.

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SUPPORTING INFORMATION

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