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RESEARCH

Early detection of rare and elusive endangered species using environmental DNA: a case study for the Eurasian otter and the white-clawed crayfish in northwestern Italy

Lorenzo Ballini[1] · Dario Ottonello[2] · Valentina Repetto[2] · Chiara Natali[1] · Giacomo Chini[1] · Livia Tolve[1] · Claudio Ciofi[1] · Sara Fratini[1] · Alessio Iannucci[1]

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Abstract
Monitoring, management and conservation of rare and elusive species often requires early detection of individuals, especially for re-introduced and endangered taxa. Environmental DNA (eDNA) approaches can enhance the detection power of traditional biomonitoring methods for low-density, newly-established populations. In this study, we used species-specific Real Time PCR TaqMan assays to assess the presence of two endangered freshwater species, the white-clawed crayfish *Austropotamobius pallipes* and the Eurasian otter *Lutra lutra* at eight sites in four river catchments in Liguria (northwestern Italy). The Eurasian otter was considered extinct in the study area since the 1980s. However, recent, although scattered sightings indicated a recolonisation by a few individuals. The white-clawed crayfish populations declined drastically and became increasingly dispersed in the western part of Liguria. Our eDNA analysis confirmed the presence of both species in some of the selected rivers and detected Eurasian otter DNA where the species was not recorded through traditional monitoring methods. This study confirms eDNA-based monitoring approaches as valuable tools to assess the presence of rare and elusive species and help implement protection plans at a local scale.

Keywords eDNA · *Lutra lutra* · *Austropotamobius pallipes* · Real time PCR · Biodiversity monitoring · Conservation

Introduction

Biodiversity monitoring is an essential tool to understand species distribution and inform wildlife conservation strategies. The effective management of rare and elusive species often requires early detection of populations, especially for reintroduced and endangered taxa (Maxwell and Jennings 2005; Jerde et al. 2011; Deiner et al. 2021; Peralta et al. 2023). The analysis of environmental DNA (eDNA) can complement traditional biomonitoring methods resulting in a more detailed description of community composition, especially in aquatic environments (Tsuji et al. 2019).

Monitoring methods based on eDNA allow for early detection of populations in which individuals are either at low densities, early ontogenetic or cryptic stages. eDNA-based techniques can help identify different species from a single sample (metabarcoding eDNA) or record the presence of a species of interest (targeted eDNA). In particular, probe-based Real Time PCR is now widely used to detect presence of a target species in an eDNA sample (Taberlet et al. 2018; Xia et al. 2018; Pawlowski et al. 2020).

Inland waters and freshwater ecosystems are among the most threatened environments and are experiencing high rates of biodiversity decline (Dudgeon 2019). The International Union for Conservation of Nature (IUCN) Red List reports that almost 22% of the known freshwater species are threatened (IUCN 2023; William-Subiza and Epele 2021). Two iconic endangered Italian native

✉ Sara Fratini
sara.fratini@unifi.it

[1] Department of Biology, University of Florence, Via Madonna del Piano 6, Sesto Fiorentino (FI) 50019, Italy

[2] ARPAL, Regional Agency for the Protection of the Ligurian Environment, Via Bombrini 8, Genoa 16149, Italy

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freshwater species are the Eurasian otter (*Lutra lutra* Linnaeus, 1758) and the white-clawed crayfish (*Austropotamobius pallipes* species complex Lereboullet, 1858).

Despite being found in a wide variety of aquatic environments in Asia, Europe and North Africa, the Eurasian otter is listed as Near-Threatened in the IUCN Red List. This species is listed as Endangered in Italy (Loy et al. 2020), where populations have been steadily declining since the last century due to habitat reduction and degradation (Loy 2018). Thanks to a number of conservation actions, otter populations are now recovering in some European countries, including southern and central Italy (Elmeros et al. 2006; Prigioni et al. 2007; Loy 2018; Bolinesi et al. 2019; Buglione et al. 2020a, b; Gaudiano et al. 2023). Recently, the presence of *L. lutra* has also been reported in northern Italian regions (Friuli Venezia Giulia, Veneto, Trentino Alto Adige and Liguria), probably as a result of recolonisation from neighbouring countries (Righetti 2011; Pavanello et al. 2015; Malthieux 2020; Nadai et al. 2022).

The white-clawed crayfish is threatened by habitat modification, pollution, competition with invasive alien species and the onset of lethal diseases (Holdich 2003). Many countries, including Italy, have experienced a massive population decline over the last few decades, so that this species has been listed as Endangered in the IUCN Red List since 2010 (Füreder et al. 2010). Italian populations of the white-clawed crayfish belong to two distinct species: *A. pallipes*, which occurs in the western part of Liguria, and *A. italicus* (including four subspecies, see Fratini et al. 2005), which is present in the rest of the Italian peninsula.

In this study, we used a Real Time PCR Taqman assay to assess the presence of the white-clawed crayfish and the Eurasian otter in four river catchments in Liguria (northwestern Italy). The Eurasian otter has been considered extinct in this region since the 1980s (see Prigioni et al. 2007). However, an isolated population was recently recorded in adjacent areas (Malthieux 2020). The white-clawed crayfish populations have declined drastically in numbers and have become increasingly dispersed in western Liguria. In this context, an eDNA-based monitoring approach is an ideal, non-invasive tool to detect the presence of both species which presumably occur in the area at low-densities. Our study aims at defining the current distribution range of *L. lutra* and *A. pallipes* in western Liguria and informing future management and conservation plans.

Materials and methods

Study area and sampling

The study area included four river catchments in western Liguria (Italy): Roia-Bevera, Argentina, Nervia and Tanaro (Fig. 1). The Tanaro basin is located at the border of the Padano-Venetian ichthyogeographic district, while the other three catchments belong to the Tuscano-Latium ichthyogeographic zone. The Roia-Bevera basin is partially in French territory (Bianco 1990, 1995). Sampling was conducted in October 2022 in eight sites, two along the Roia river (ROI1 and ROI2), two in the Bevera river (BEV1 and BEV2), and one along the Argentina (ARG2), Tanaro (TAN), Carpasina (CAR) and Nervia (NER2) rivers (Fig. 1). Rivers and sampling sites were selected based on occurrence of *L. lutra* and *A. pallipes* recorded during previous monitoring campaigns (see Salvidio et al. 2002; Bologna and Cristiani 2012; Capurro et al. 2015; Malthieux 2020; Ottonello pers. obs.). Moreover, the study area represents the most direct route for recolonisation by otter populations from France (Malthieux 2020).

Water samples were collected at each site in six spatial replicates randomly selected on the two banks and in the middle of the river along a 150 m long transect. Each replicate consisted of one litre of water collected using plastic jars previously sterilised with sodium hypochlorite. Water samples were filtered on-site using a portable hand vacuum-pump connected to a polypropylene flask (Thermo Fisher Scientific). We used sterile disposable filter units with nitrocellulose membrane and a pore size of 0.2 µm (Thermo Fisher Scientific). All filters were immediately preserved in absolute ethanol upon water collection and stored at -20 °C prior to DNA extraction. A jar containing one litre of DNA-free deionized sterile water was left opened at each site for two minutes. The water was then filtered on-site and used as negative field control.

DNA extraction

Environmental DNA purification was carried out in a laminar flow cabinet using sterile equipment to avoid exogeneous DNA contamination. eDNA was extracted from nitrocellulose membrane filters using the ZymoBIOMICS DNA Miniprep Kit (Zymo Research) according to the manufacturer's guidelines, eluted in a final volume of 50 µL of sterilised water and preserved at -20 °C.

To verify that DNA extraction from water samples provided sufficient metazoan genetic material, we measured

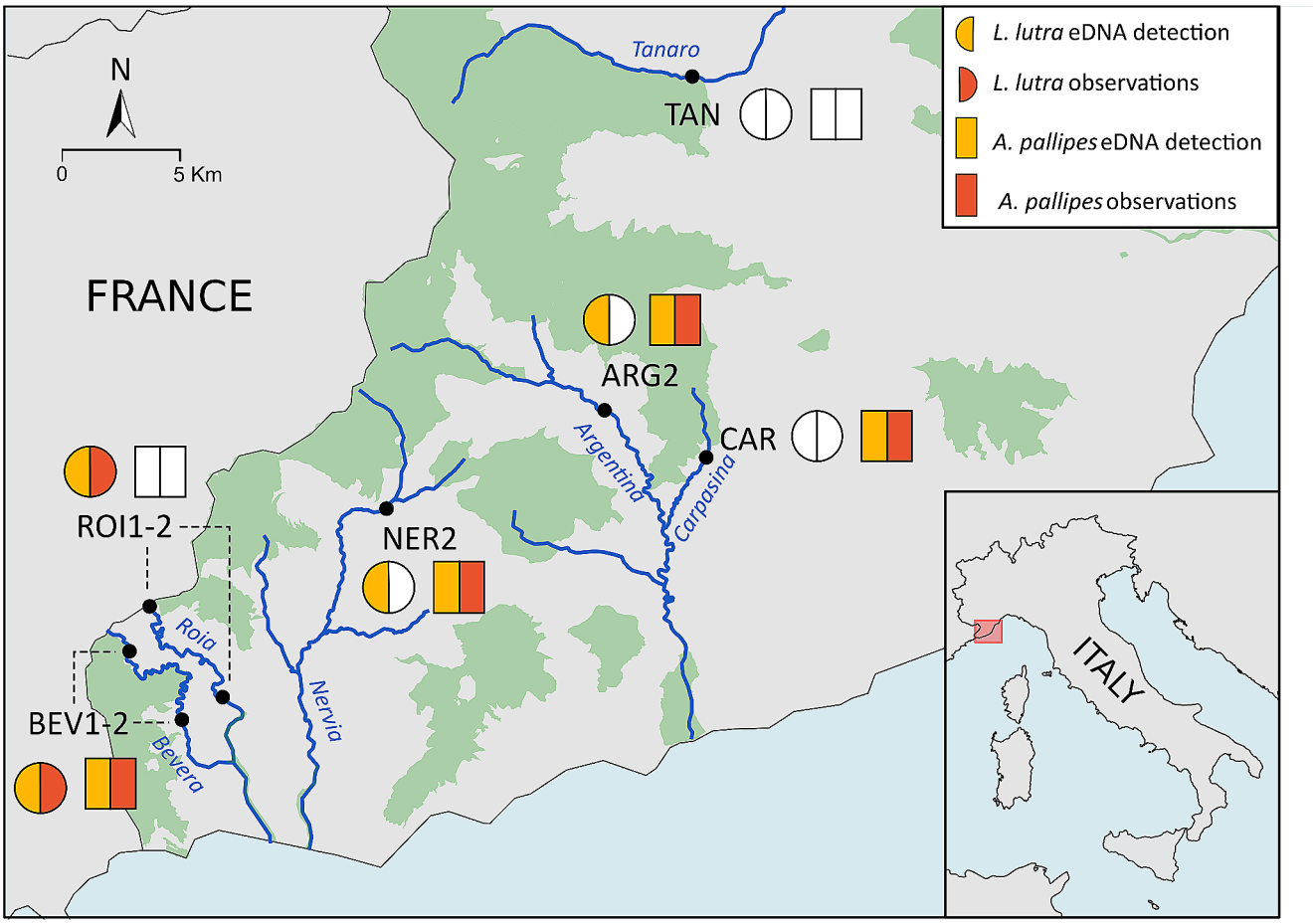

Fig. 1 Map of the study area. Environmental DNA detection results and recent observation data are shown for each river. Bullets show locations of sampling sites. White semicircles and rectangles indicate lack of detection or observations. Dark green shading shows Natura 2000 Network areas

DNA concentrations using the Qubit dsDNA HS Assay Kit (Invitrogen) and amplified two universal mitochondrial DNA (mtDNA) metabarcode markers using PCR primers Tele02 and Vert01 (Riaz et al. 2011; Taberlet et al. 2018). Amplification reactions were performed using 1X Invitrogen *Taq* DNA Polymerase PCR Buffer, 1 U of *Taq* DNA Polymerase (Thermo Fisher Scientific), 2 mM $MgCl_2$, 0.3 mM dNTPs, 0.5 µM of each primer and 1 µL of sample DNA in 25 µL total volume. The following amplification conditions were used: 5 min at 94 °C, 35 cycles of 94 °C for 30 s, 54 °C (Tele02) or 49 °C (Vert01) for 30 s, 72 °C for 1 min and a final extension step at 72 °C for 10 min. PCR products were resolved on a 1.2% agarose gel stained with GelRed Nucleic Acid Gel Stain (Biotum). DNA-free ddH_2O and *L. lutra* DNA extracted from fresh biological material were included in each PCR reaction as negative and positive controls, respectively.

Real time PCR assay

Presence of target species in the eDNA samples was detected using species-specific probe-based TaqMan assays. For *L. lutra*, we used the assay reported by Thomsen et al. (2012) which amplifies a 80 bp long fragment of the mtDNA cytochrome b gene. For *A. pallipes*, we used the assay reported by Troth et al. (2020) which amplifies a 109 bp fragment of the mtDNA cytochrome c oxidase subunit I gene. Each assay included two PCR primers and a target-specific dual labelled probe with fluorescent reporter and non-fluorescent quencher. A ROX fluorescent dye included in the Master Mix was used as an internal passive reference to normalize PCR fluorescent dye signals.

Positive controls consisted of *L. lutra* and *A. pallipes* DNA extracted from ethanol-preserved samples from the Natural History Museum of the University of Florence, Italy. For the white-clawed crayfish, we included positive controls of *A. pallipes* and three subspecies of *A. italicus* (*A. i. carinthiacus, A. i. italicus and A. i. meridionalis*).

DNA extractions were performed from muscle tissues using the PureLink Genomic DNA Mini Kit (Thermo Fisher Scientific) following the manufacturer's protocol.

Real Time PCR assays were performed on a QuantStudio 7 Flex Real-Time PCR System (Life Technologies). Ten-fold serial dilutions of positive control DNA were tested in ten replicates to assess Real Time PCR amplification efficiency and define the limit of detection (LOD, expressed as threshold cycle Ct) as reported in Bustin et al. (2009); Klymus et al. (2020a, b). Amplification conditions were as follows: a Pre-Read Stage at 60 °C for 30 s, hold at 95 °C for 20 s, 55 cycles at 95 °C for 1 s and 52 °C for 20 s followed by a Post-Read Stage at 60 °C for 30 s. Amplification reactions were conducted in a total volume of 20 µL containing 1X TaqMan Fast Advanced Master Mix (Thermo Fisher Scientific), 0.5 µM of each primer, 0.25 µM TaqMan probe and 5 µL template DNA.

For each sampling site, eDNA samples, positive and negative controls were amplified in technical triplicate in the same PCR run. Negative field controls were processed as eDNA samples. A Real Time PCR replicate was recorded as positive if Ct was lower than the LOD, it had a uniform curve morphology and no amplification occurred in the negative template and field controls (Bustin et al. 2009; Ficetola et al. 2015; Klymus et al. 2020a, b). Following the criteria proposed by Taberlet et al. (1996), Ficetola et al. (2015), Buxton et al. (2021) and Sanz et al. (2023) to avoid false positive and negative results, the presence of a species was considered as ascertained in the case of two positive amplifications out of the total of technical and spatial replicates.

Results and discussion

Environmental DNA concentrations ranged from 0.075 to 36.6 ng μL^{-1}. The Tele02 and Vert01 PCR assays confirmed successful extraction of metazoan DNA from the eDNA samples for we obtained a visible band for each sample when loaded on an agarose gel. Negative PCR controls excluded the possibility of false-positive amplification due to human DNA contamination.

For both detection assays, the amplification efficiency of Real Time PCR estimated by means of calibration curves determined using serial dilutions of the positive controls was about 90% (*L. lutra*: $y = -3.63x + 24.05$, $r^2 = 0.99$; A. *pallipes*: $y = -3.5x + 25.68$, $r^2 = 0.97$). The LOD corresponded to a Ct value of 43.7 ± 0.1 for the Eurasian otter assay and a Ct value of 43.3 ± 0.3 for the white-clawed crayfish assay. Ct values of positive Real Time PCR amplifications ranged from 37.7 to 43.5 for the otter assay and from 36.7 to 40.2 for the crayfish assay (Table 1).

Negative field and PCR controls produced no amplification, while PCR products of positive controls confirmed the reliability of the selected assays. For the white-clawed crayfish, we obtained a positive amplification for *A. pallipes* and the three tested subspecies of *A. italicus*. This result indicated that the sensitivity of the assay did not allow for the distinction between species and subspecies belonging to the *A. pallipes* species complex.

We recorded the presence of the Eurasian otter in four out of the six investigated rivers (Roia, Bevera, Argentina and Nervia). No presence of *L. lutra* was recorded in the Tanaro and Carpasina rivers. We detected the presence of the white-clawed crayfish in all river streams but the Tanaro and Roia rivers (Fig. 1; Table 1).

Historical sightings of the Eurasian otter in the western part of Liguria suggested the presence of few individuals in the high valley of the Tanaro, Argentina, Nervia, Roia and Bevera rivers (Balletto 1977; Vigna Taglianti and Bologna 1982). The species was considered extinct in the area ever since (Prigioni et al. 2007; Bologna and Cristiani 2012). Traces of *L. lutra* have been recently recorded upstream of BEV1-2 and ROI1-2 sampling sites in the Roia and Bevera rivers, indicating the presence of a relict population (Malthieux 2020). Our eDNA analysis confirmed the presence of the Eurasian otter on the Italian side of these rivers and detected the presence of *L. lutra* in adjacent basins where it was not previously observed (i.e., the Argentina and Nervia rivers).

The presence of the white-clawed crayfish was reported in five out of the six rivers included in this study, that is the Bevera, Nervia, Argentina, Carpasina (Capurro et al. 2015) and Tanaro (Salvidio et al. 2002). Recent observations, however, confirmed the presence of *A. pallipes* in all the above rivers but the Tanaro. Our eDNA approach confirmed recent surveys as we recorded the occurrence of the white-clawed crayfish only in BEV1, NER2, CAR and ARG2.

The majority of sites where we detected the presence of our target species are in close proximity to (NER2, ARG2, CAR) or mark the boundaries (BEV1-2) of Natura 2000 sites (Fig. 1). None of these Natura 2000 sites report *L. lutra* in their Standard Data Forms (SDF), while *A. pallipes* is only listed in the SDF of the protected areas established in the upper parts of the Carpasina and Argentina rivers.

This study confirms that eDNA-based monitoring approaches enhance the detection power of traditional biomonitoring surveys and are valuable tools to inform efficient management and protection schemes for threatened species (Pascher et al. 2022). Further investigations

Table 1 Real Time PCR Ct values for eDNA samples tested using *L. lutra* and *A. pallipes* complex Taqman assays. Ct values of the three technical replicates are reported for each sample. Samples that showed no amplification are marked as NA (No Amplification). Ct values of amplifications scored as positive according to assignation criteria are highlighted in bold

River	Sampling site ID	Sample ID	Ct Lutra lutra			Ct Austropotamobius pallipes		
Argentina	ARG2	ARG2_NEG	NA	NA	NA	NA	NA	NA
		ARG2_1	NA	NA	NA	NA	NA	NA
		ARG2_2	NA	NA	NA	NA	NA	NA
		ARG2_3	NA	NA	NA	NA	NA	**40.1**
		ARG2_4	NA	NA	NA	NA	NA	NA
		ARG2_5	NA	**39.5**	**40.0**	NA	NA	NA
		ARG2_6	NA	NA	NA	NA	**39.6**	NA
Roia	ROI1	ROI1_NEG	NA	NA	NA	NA	NA	NA
		ROI1_1	NA	NA	NA	NA	NA	NA
		ROI1_2	NA	NA	NA	NA	NA	NA
		ROI1_3	**42.7**	NA	NA	NA	NA	NA
		ROI1_4	NA	NA	NA	NA	NA	NA
		ROI1_5	NA	NA	NA	NA	NA	NA
		ROI1_6	NA	NA	NA	NA	NA	NA
	ROI2	ROI2_NEG	NA	NA	NA	NA	NA	NA
		ROI2_1	NA	NA	NA	NA	NA	NA
		ROI2_2	NA	NA	NA	NA	NA	NA
		ROI2_3	NA	NA	NA	NA	NA	NA
		ROI2_4	NA	NA	**43.5**	NA	NA	NA
		ROI2_5	NA	NA	NA	NA	NA	NA
		ROI2_6	NA	NA	NA	NA	NA	NA
Tanaro	TAN	TAN_NEG	NA	NA	NA	NA	NA	NA
		TAN_1	NA	NA	NA	NA	NA	NA
		TAN_2	NA	NA	NA	NA	NA	NA
		TAN_3	NA	NA	NA	NA	NA	NA
		TAN_4	NA	NA	NA	NA	NA	NA
		TAN_5	NA	NA	NA	NA	NA	NA
		TAN_6	NA	NA	NA	NA	NA	NA
Carpasina	CAR	CAR_NEG	NA	NA	NA	NA	NA	NA
		CAR_1	NA	NA	NA	**38.1**	**39.4**	NA
		CAR_2	NA	NA	NA	NA	NA	NA
		CAR_3	NA	NA	NA	NA	NA	NA
		CAR_4	NA	NA	NA	NA	**40.2**	NA
		CAR_5	NA	NA	NA	NA	NA	**39.0**
		CAR_6	NA	NA	NA	NA	NA	NA
Bevera	BEV1	BEV1_NEG	NA	NA	NA	NA	NA	NA
		BEV1_1	NA	**37.7**	NA	**37.2**	NA	NA
		BEV1_2	NA	NA	NA	NA	NA	NA
		BEV1_3	**38.3**	NA	NA	NA	**37.5**	NA
		BEV1_4	NA	NA	NA	NA	NA	NA
		BEV1_5	NA	NA	NA	NA	NA	**36.7**
		BEV1_6	NA	NA	NA	NA	NA	NA
	BEV2	BEV2_NEG	NA	NA	NA	NA	NA	NA
		BEV2_1	NA	NA	NA	NA	NA	NA
		BEV2_2	NA	NA	NA	NA	NA	NA
		BEV2_3	NA	NA	NA	NA	NA	NA
		BEV2_4	NA	NA	NA	NA	NA	NA
		BEV2_5	NA	NA	NA	NA	NA	NA
		BEV2_6	NA	NA	NA	NA	NA	NA
Nervia	NER2	NER2_NEG	NA	NA	NA	NA	NA	NA
		NER2_1	NA	NA	NA	NA	**36.9**	NA
		NER2_2	NA	NA	NA	NA	NA	NA
		NER2_3	**42.8**	**41.6**	NA	NA	NA	NA

Table 1 (continued)

River	Sampling site ID	Sample ID	Ct Lutra lutra			Ct Austropotamobius pallipes		
		NER2_4	NA	NA	NA	NA	NA	NA
		NER2_5	NA	NA	NA	**38.9**	NA	NA
		NER2_6	NA	NA	NA	NA	NA	NA

are needed to define in detail the distribution range of the target species in western Italy and reconstruct the recolonization routes of *L. lutra* of the Italian watersheds. Moreover, additional studies are needed to understand whether the presence of the Eurasian otter in western Italy is occasional or the species is reestablishing well-structured and viable populations.

Author contributions L.B., S.F. and A.I. wrote the manuscript. S.F, C.C., D.O., V.R., and A.I. conceptualized and designed the study. L.B., L.T. and G.C. collected the samples. L.B., C.N., A.I and S.F. performed lab work and provided lab support. All authors read and approved the final version of the manuscript.

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Data availability The data generated and/or analysed during the current study are available from the corresponding author on reasonable request.

Declarations

Competing interests The authors declare no competing interests.

References

Balletto E (1977) Analisi faunistico-venatoria ed ecologica della Regione Liguria. Tipografia Don Bosco, Genova

Bianco PG (1990) Potential role of the palaeohistory of the Mediterranean and paratethys basins on the early dispersal of Euro-Mediterranean freshwater fishes. Ichthyol Explor Freshw 1:167–184

Bianco PG (1995) Mediterranean endemic freshwater fishes of Italy. Biol Conserv 72(2):159–170. https://doi.org/10.1016/0006-3207(94)00078-5

Bolinesi F, Viglietti S, Maio N, Guarino FM (2019) Presence of the eurasian otter *Lutra lutra* (Linnaeus, 1758) (Mammalia Mustelidae) in the Foce Sele-Tanagro Nature Reserve (Campania, Southern Italy). Biodivers J 10(2):121–126. https://doi.org/10.31396/Biodiv.Jour.2019.10.2.121.126

Bologna MA, Cristiani G (2012) Contributo alla teriofauna dell'atra Val Tanaro, Alpi Liguri (CN-IM). Riv Piemont Stor Nat 33:295–319

Buglione M, Petrelli S, Troiano C, Notomista T, Petrella A, De Riso L, Poerio L, Cascini V, Bartolomei R, Fulgione D (2020a) Spatial genetic structure in the eurasian otter (L*utra lutra*) meta-population from its core range in Italy. Contrib Zool 90(1):70–92. https://doi.org/10.1163/18759866-BJA10012

Buglione M, Petrelli S, Troiano C, Notomista T, Rivieccio E, Fulgione D (2020b) The diet of otters (*Lutra lutra*) on the Agri river system, one of the most important presence sites in Italy: a molecular approach. PeerJ 8:e9606. https://doi.org/10.7717/peerj.9606

Bustin SA, Benes V, Garson JA, Hellemans J, Huggett J, Kubista M, Mueller R, Nolan T, Pfaffl MW, Shipley GL, Vandesompele J, Wittwer CT (2009) The MIQE guidelines: minimum information for publication of quantitative real-time PCR experiments. Clin Chem 55(4):611–622. https://doi.org/10.1373/clinchem.2008.112797

Buxton A, Matechou E, Griffin J, Diana A, Griffiths RA (2021) Optimising sampling and analysis protocols in environmental DNA studies. Sci Rep 11(1):11637. https://doi.org/10.1038/s41598-021-91166-7

Capurro M, Oneto F, Ottonello D, Pericci R, Mignone W, Riina MV, Acuti PL (2015) Nota sulla presenza di esemplari xantocroici di gambero di fiume *Austropotamobius pallipes* specie complex (Lerebouillet, 1858) in Liguria (Italia nord-occidentale). Ann Mus Civ Stor Nat G Doria VIII:1–12

Deiner K, Yamanaka H, Bernatchez L (2021) The future of biodiversity monitoring and conservation utilizing environmental DNA. Environ DNA 3:3–7. https://doi.org/10.1002/edn3.178

Dudgeon D (2019) Multiple threats imperil freshwater biodiversity in the Anthropocene. Curr Biol 29(19):R960–R967. https://doi.org/10.1016/j.cub.2019.08.002

Elmeros M, Hammershoj M, Madsen A, Sogaard B (2006) Recovery of the otter *Lutra lutra* in Denmark monitored by field surveys and collection of carcasses. Hystrix 17(1):17–28. https://doi.org/10.4404/hystrix-17.1-4361

Ficetola GF, Pansu J, Bonin A, Coissac E, Giguet-Covex C, De Barba M, Gielly L, Lopes CM, Boyer F, Pompanon F, Rayé G, Taberlet P (2015) Replication levels, false presences and the estimation of the presence/absence from eDNA metabarcoding data. Mol Ecol Resour 15(3):543–556. https://doi.org/10.1111/1755-0998.12338

Fratini S, Zaccara S, Barbaresi S, Grandjean F (2005) Phylogeography of the threatened crayfish (genus *Austropotamobius*) in Italy: implications for its taxonomy and conservation. Heredity 94(1):108–118. https://doi.org/10.1038/sj.hdy.6800581

Füreder L, Gherardi F, Holdich D, Reynolds J, Sibley P, Souty-Grosset C (2010) In: IUCN 2010. The IUCN Red List of Threatened Species 2010.3: e.T2430A9438817

Gaudiano L, Cascella A, L'Erario P, Corriero G (2023) First data on the presence of Lutra lutra in Bosco Incoronata Natural Regional Park (Apulia, South Italy). BORNH Bull Reg Nat Hist 3(1):1–11. https://doi.org/10.6093/2724-4393/9985

Holdich D (2003) Ecology of the White-clawed Crayfish. Conserving Natura 2000 Rivers Ecology Series No. 1. English Nature, Peterborough

IUCN (2023) The IUCN Red list of threatened species. Version 2022-2. https://www.iucnredlist.org

Jerde CL, Mahon AR, Chadderton WL, Lodge DM (2011) Sight-unseen detection of rare aquatic species using environmental DNA: eDNA surveillance of rare aquatic species. Conserv Lett 4(2):150–157. https://doi.org/10.1111/j.1755-263X.2010.00158.x

Klymus KE, Merkes CM, Allison MJ, Goldberg CS, Helbing CC, Hunter ME, Jackson CA, Lance RF, Mangan AM, Monroe EM, Piaggio AJ, Stokdyk JP, Wilson CC, Rithcer CA (2020a) Reporting the limits of detection and quantification for environmental DNA assays. Environ DNA 2:271–282. https://doi.org/10.1002/edn3.29

Klymus KE, Ruiz Ramos DV, Thompson NL, Richter CA (2020b) Development and testing of species-specific quantitative PCR assays for environmental DNA applications. J Vis Exp 165:e61825. https://doi.org/10.3791/61825

Loy A (2018) Eurasian otter. Duplaix N. Savage M. The global Otter Conservation Strategy. IUCN/SSC Otter Specialist Group, Salem, Oregon, pp 46–56

Loy A, Kranz A, Oleynikov A, Roos A, Savage M, Duplaix N (2020) *Lutra lutra*. In IUCN 2020. The IUCN Red List of Threatened Species 2022: e.T12419A218069689

Malthieux L (2020) La Loutre d'Europe *Lutra lutra* (Linnaeus, 1758) en Roya- Bévéra: relique ou retour ? Prospections, état des lieux et implications. Faune-PACA Publication 98:22

Maxwell D, Jennings S (2005) Power of monitoring programmes to detect decline and recovery of rare and vulnerable fish. J Appl Ecol 42(1):25–37. https://doi.org/10.1111/j.1365-2664.2005.01000.x

Nadai GD, Cassol M, Lapini L (2022) First data on the natural recovery of the Eurasian otter (*Lutra lutra* Linnaeus, 1758) in Veneto Region (north-eastern Italy). Habitat Online. https://www.habitatonline.eu/2022/11/first-data-on-the-natural-recovery-of-the-eurasian-otter-lutra-l-lutra-linnaeus-1758-in-veneto-region-north-eastern-italy/

Pascher K, Švara V, Jungmeier M (2022) Environmental DNA-based methods in biodiversity monitoring of protected areas: application range, limitations, and needs. Diversity 14(6):463. https://doi.org/10.3390/d14060463

Pavanello M, Lapini L, Kranz A, Iordan F (2015) Rediscovering the eurasian otter (*Lutra lutra* L.) in Friuli Venezia Giulia and notes on its possible expansion in northern Italy. IUCN Otter Specialist Group Bull 32(1):12–20

Pawlowski J, Apothéloz-Perret-Gentil L, Mächler E, Altermatt F (2020) Environmental DNA applications for biomonitoring and bioassessment in aquatic ecosystems. Guidelines. Bern, Switzerland, Federal Office for the Environment. https://doi.org/10.5167/UZH-187800

Peralta D, Vaz-Freire T, Ferreira C, Mendes T, Mira A, Santos S, Alves PC, Lambin X, Beja P, Paupério J, Pita R (2023) From species detection to population size indexing: the use of sign surveys for monitoring a rare and otherwise elusive small mammal. Eur J Wildl Res 69:9. https://doi.org/10.1007/s10344-022-01634-2

Prigioni C, Balestrieri A, Remonti L (2007) Decline and recovery in otter *Lutra lutra* populations in Italy. Mammal Rev 37(1):71–79. https://doi.org/10.1111/j.1365-2907.2007.00105.x

Riaz T, Shehzad W, Viari A, Pompanon F, Taberlet P, Coissac E (2011) ecoPrimers: inference of new DNA barcode markers from whole genome sequence analysis. Nucleic Acids Res 39(21):e145. https://doi.org/10.1093/nar/gkr732

Righetti D (2011) Return of the otter in South Tyrol (NE Italy). In: Prigioni C, Loy A, Balestrieri A, Remonti L (eds) Proceedings of the IUCN XI International Otter Colloquium. Hystrix It J Mamm Supp. https://doi.org/10.4404/hystrix-22.0-4744

Salvidio S, Mori M, Lattes A, Galli L, Arillo A (2002) The freshwater crayfish *Austropotamobius pallipes* (Lereboullet, 1858) in Liguria, NW Italy: implications for management at the regional level. Bull Fr Pêche Piscic 367:663–670. https://doi.org/10.1051/kmae:2002057

Sanz N, Franch N, Araguas RM, Viñas J, Vidal O (2023) Environmental DNA assay for the detection of the American Bullfrog (*Lithobates catesbeianus*) in the early stages of the Invasion in the Ebre Delta. Animals 13(4):683. https://doi.org/10.3390/ani13040683

Taberlet P, Griffin S, Goossens B, Questiau S, Manceau V, Escaravage N, Waits LP, Bouvet J (1996) Reliable genotyping of samples with very low DNA quantities using PCR. Nucleic Acids Res 24(16):3189–3194. https://doi.org/10.1093/nar/24.16.3189

Taberlet P, Bonin A, Zinger L, Coissac E (2018) Environmental DNA: for Biodiversity Research and Monitoring. Oxford University Press, Oxford

Thomsen PF, Kielgast J, Iversen LL, Wiuf c, Rasmussen M, Gilbert MT, Orlando L, Willerslev E (2012) Monitoring endangered freshwater biodiversity using environmental DNA: species monitoring by environmental DNA. Mol Ecol 21(11):2565–2573. https://doi.org/10.1111/j.1365-294X.2011.05418.x

Troth CR, Burian A, Mauvisseau Q, Bulling M, Nigthingale J, Mauvisseau C, Sweet MJ (2020) Development and application of eDNA-based tools for the conservation of white-clawed crayfish. Sci Total Environ 748:141394. https://doi.org/10.1016/j.scitotenv.2020.141394

Tsuji S, Takahara T, Doi H, Shibata N, Yamanaka H (2019) The detection of aquatic macroorganisms using environmental DNA analysis - A review of methods for collection, extraction, and detection. Environ DNA 1:99–108. https://doi.org/10.1002/edn3.21

Vigna Taglianti A, Bologna MA (1982) La fauna. AA.VV. Piano per Il Parco Naturale Regionale Delle Alpi Liguri. Sistemi I, II, III, Regione Liguria

Williams-Subiza EA, Epele LB (2021) Drivers of biodiversity loss in freshwater environments: a bibliometric analysis of the recent literature. Aquat Conserv: Mar Freshw Ecosyst 31(9):2469–2480. https://doi.org/10.1002/aqc.3627

Xia Z, Johansson ML, Gao Y, Zhan L, Haffner GD, MacIsaac, Zhan A (2018) Conventional versus real-time quantitative PCR for rare species detection. Ecol Evol 8(23):11799–11807. https://doi.org/10.1002/ece3.4636