Journal of Invertebrate Pathology 201 (2023) 108026

Contents lists available at ScienceDirect

Journal of Invertebrate Pathology

journal homepage: www.elsevier.com/locate/jip

eDNA monitoring as a tool for evaluating the reintroduction of *Austropotamobius pallipes* after a crayfish plague outbreak

Gloria Casabella-Herrero, Laura Martín-Torrijos, Javier Diéguez-Uribeondo *

Mycology Department, Real Jardín Botánico-CSIC, Plaza Murillo 2, 28014 Madrid, Spain

ARTICLE INFO

Keywords:
Invasive Alien Species
Aphanomyces astaci
eDNA monitoring
Endangered species conservation
Native species reintroduction

ABSTRACT

The crayfish plague, a severe disease caused by the oomycete *Aphanomyces astaci*, is responsible for most population declines of susceptible crayfish in Europe. This pathogen has been devastating native populations of *Austropotamobius pallipes* since the 1970s in the Iberian Peninsula. In this study, we report a massive mortality event in one of the most important Spanish populations of *A. pallipes*. We aimed to: (i) identify the cause of the mortality, and (ii) evaluate the reintroduction viability of the species. Over the course of six months, we used environmental DNA (eDNA) and traditional trap-based methods to detect the presence of *A. astaci* or of native or invasive crayfish in order to evaluate the reintroduction viability of *A. pallipes* to the affected population. We did not capture any live crayfish or detect the presence of *A. astaci* in the reservoir water during the six months following the mass mortality event. Our analyses indicated that it was feasible to initiate a reintroduction program at the site, which will continue to be monitored for three to five years and will help improve the conservation status of *A. pallipes*.

1. Introduction

The management of freshwater species populations is one of the most relevant conservation challenges over the last decades due to their constant decline. Between 1970 and 2014, the global populations of freshwater species dropped by an estimated 83 % owing to different factors, including climate change, freshwater salinization and microplastic pollution (Reid et al., 2019). However, one of the most important threats to freshwater species are invasive alien species (IAS), and their role as vectors for the introduction of emerging diseases (Conn, 2014, Reid et al., 2019). Freshwater IAS severely impact community structure and ecosystem functioning (Havel et al., 2015), and emerging diseases pose severe threats to wildlife and human health, as well as to food and natural resources security (Fisher et al., 2012, Voyles et al., 2015). One of the most significant emerging diseases translocated via IAS, and affecting freshwater ecosystems and wildlife is the crayfish plague. This disease is caused by the oomycete *Aphanomyces astaci*, a pathogen that originates from North America (Martín-Torrijos et al., 2021), and is specialized in parasitizing freshwater decapods (Rezinciuc et al., 2015). Outside their native range, both North American crayfish and the pathogen act as IAS. The damaging impact of the pathogen has been so great that *A. astaci* is one of the few microorganisms included in the 100 world's worst invasive alien species list (Lowe et al., 2004). The pathogen has been translocated and introduced worldwide alongside its vectors, North American crayfish, and has caused severe damage to freshwater environments (reviewed in Rezinciuc et al., 2015, Jussila et al., 2021). In Europe, the introduction of *A. astaci* has caused mass mortalities and, consequently, drastic population declines of susceptible native species since the 19th century (Rezinciuc et al., 2015).

Until recently, most methods for identifying *A. astaci* infection required the analyses of crayfish after their death. Initially, the crayfish plague could only be diagnosed by microscopic observation and attempting culture isolation (reviewed in Rezinciuc et al., 2015). The latter development of molecular techniques has provided several pure-culture independent diagnostic methods (*e.g.*, Oidtmann et al., 2004, 2006, Vrålstad et al., 2009), and has allowed the description of the genetic diversity of the pathogen (*e.g.*, Huang et al., 1994, Makkonen et al., 2012, Grandjean et al., 2014, Makkonen et al., 2018). To date, 13 *A. astaci* haplotypes have been described from Europe, Japan and the south-eastern USA using the mitochondrial (mtDNA) ribosomal small

Abbreviations: IAS, invasive alien species; nrITS, nuclear ribosomal internal transcribed spacer; BI, Bayesian inference; ML, maximum likelihood; mtDNA, mitochondrial DNA; rrnS, ribosomal small subunit; rrnL, ribosomal large subunit; qPCR, quantitative PCR; eDNA, environmental DNA.

* Corresponding author.
E-mail addresses: gcasabella@rjb.csic.es (G. Casabella-Herrero), lmtorrijos@rjb.csic.es (L. Martín-Torrijos), dieguez@rjb.csic.es (J. Diéguez-Uribeondo).

https://doi.org/10.1016/j.jip.2023.108026
Received 26 April 2023; Received in revised form 20 November 2023; Accepted 22 November 2023
Available online 24 November 2023

(rrnS) and large (rrnL) subunit regions (Makkonen et al., 2018, Martín-Torrijos et al., 2018, Martín-Torrijos et al., 2021, Martín-Torrijos et al., 2023). The haplotypes reported from Europe (*i.e.*, a, b, d1, d2 and e-haplotypes) exhibit a species-specific pattern of distribution, *e.g.*, b-haplotype corresponds to *Pacifastacus leniusculus*, d1- and d2-haplotypes to *Procambarus clarkii,* and e-haplotype corresponds to *Faxonius limosus*. This pattern allows the occurrence of the pathogen to be associated with specific introductions of North American crayfish species in Europe.

The development of environmental DNA (eDNA) sampling techniques has been changing the concept of species sampling and monitoring by enabling detection directly from the environment. This approach has proven useful for field detection of hard to detect taxa in freshwater systems, *e.g.*, IAS during the first invasion steps (Larson et al., 2020, Morisette et al., 2017), endangered or presumed locally extinct native species (Rees et al., 2014, Sigsgaard et al., 2015, Goldberg et al., 2016), or emerging diseases (Gomes et al., 2017, Sieber et al., 2020), providing a suitable framework for crayfish plague monitoring. Strand et al., (2012, 2014) demonstrated the detectability of *A. astaci* under both laboratory and natural conditions with eDNA, and some studies even showed more sensitivity for *A. astaci* detection with eDNA than with traditional trap-based methods (Wittwer et al., 2018, 2019). In addition, methods based on eDNA have also been widely applied for the detection of IAS and endangered crayfish (*e.g.*, Agersnap et al., 2017, Atkinson et al., 2019, Rusch et al., 2020, Troth et al., 2020, Chucholl et al., 2021), sometimes also showing higher detection rates than traditional trapping (Tréguier et al., 2014, Strand et al., 2019). The results of these studies have led to the establishment of eDNA-based monitoring programs for both *A. astaci* and crayfish by some authorities (Cheslett et al., 2019, Strand et al., 2019).

In the Iberian Peninsula, North American crayfish species, *i.e.*, *P. clarkii* and *P. leniusculus*, were first introduced in the 1970s (Habsburgo-Lorena, 1979) and subsequently caused the spread of *A. astaci* b-, d1- and d2-haplotypes throughout the Spanish basins (Martín-Torrijos et al., 2019). Crayfish translocations for leisure fishing and food consumption used to be common, which allowed the crayfish plague to reach habitats of the native species *Austropotamobius pallipes*. Native *A. pallipes* populations started suffering sudden mass mortalities of up to 100 % individuals (Diéguez-Uribeondo et al., 1997a). It was estimated that, by the beginning of the 21st century, 80 % of the populations of *A. pallipes* had been lost (Alonso et al., 2000), leading to the inclusion of the species in the Spanish National Catalogue of Threatened Species and the List of Wild Species under Special Protection (Real Decreto 139/2011, February 4th). The protection of the remaining wild populations of *A. pallipes* is a conservation priority in Spain, in line with European policies (Council Directive 92/43/CEE, of May 21st 1992). One of the most important Spanish populations of *A. pallipes* was located in the Leurtza reservoirs in Navarra. In this region, *A. pallipes* is considered a species at risk and is therefore protected (Decreto Foral 142/1996, March 11th). The Leurtza reservoirs cover an area of 8 hectares (ha), with approximately 7 ha in the lower reservoir and 1 ha in the upper reservoir. Until recently, this area possibly held the largest population of *A. pallipes* in Europe and has been a key conservation area for decades. This population has guaranteed crayfish restocking in many areas of Navarra, and scientific research on it has been conducted for, at least, the past 30 years. Unfortunately, in May 2022, a massive crayfish mortality event occurred in the Leurtza reservoirs, and the suspected cause was infection by *A. astaci*. In this study, we aimed to: (i) identify the cause and origin of the crayfish mortality using microscopy and molecular approaches, and (ii) evaluate the reintroduction viability of the species using both eDNA and traditional trap-based sampling methods to confirm the absence of *A. astaci*, crayfish IAS, and *A. pallipes* survivors prior to any reintroduction.

2. Materials and methods

2.1. Study area

The Leurtza reservoirs were built in the early 20th century for electricity production and is a recreational area regulated by the Government of Navarra (Spain) (Fig. 1A) consisting of two reservoirs that are connected and separated by concrete walls, but that maintain a water connection. Two streams flow into the upper reservoir, while the upper one flows into the lower one, and the lower one flows into a downstream drainage (Fig. 1B). Most of the *A. pallipes* population inhabited the lower reservoir as the concrete walls prevented crayfish from expanding into the upper one (Fig. 1B) (though the presence of *A. pallipes* in the upper reservoir has been occasionally detected in the past, but never in the upper streams). Until the outbreak in May 2022, the *A. pallipes* population in the lower reservoir was believed to be the largest crayfish population in Spain, with CPUE numbers that could vary from 5 to up to 600 depending of the sampling points and time of the year. Overall, the population density was estimated at approximately 12 individuals per square meter (Diéguez-Uribeondo, unpublished data). During our sampling campaigns, the two streams had low flow and a narrow channel due to high temperatures and low rainfall. We established a total of seven sampling points (SP) for water monitoring: SP1 – Stream 1, SP2 – Stream 2, SP3 – Upper reservoir (Fig. 2B and 2D), SP4 – Upper part of the lower reservoir, SP5 – Mid part of the lower reservoir, SP6 - Lower part of the lower reservoir (Fig. 2A), and SP7 – Drainage of the lower reservoir (Fig. 2C). The primary purpose of sampling SP1, SP2 and SP3 was to compare their status with that of the lower reservoir, and to try to discover any indication of the origin (and direction) of the infection into the water system. The sampling of SP3, SP4, SP5, SP6 and SP7 aimed to detect possible surviving *A. pallipes*, introduced *P. leniusculus* or remaining *A. astaci* associated with any crayfish species.

2.2. Crayfish samples

2.2.1. Microscopic and molecular analyses

In May 2022, we collected 20 dead crayfish from the lower reservoir area, specifically close to the SP3. These samples were preserved in ethanol 96° after the crayfish mortality event. We excised and cleaned the soft sub-abdominal cuticles of the specimens, and we examined the presence of hyphae of *A. astaci* using an inverted Olympus CKX41SF microscope (Olympus Optical, Tokyo, Japan). Although species of *Aphanomyces* are very difficult to distinguish by eye in the absence of reproductive structures, the presence of growth of abundant aseptate hyphae within the ventral abdominal cuticle of crayfish are suggestive of *A. astaci* infection. All samples were handled individually, and the tools were sterilized by flame before and after use, to avoid cross contamination.

After the cuticles were examined, we rinsed them three times in TE buffer (TRIS 10 mM/ EDTA 1 mM, pH 8) for at least 30 min each time to remove the ethanol and then transferred them to 2 mL Eppendorf tubes. We manually fragmented the cuticles and froze them at −80 °C overnight before homogenizing them using a TissueLyser (Qiagen, Germany). DNA was extracted from the samples using the E.Z.N.A. Insect DNA Kit (Omega Bio-tek, Norcross, Atlanta, USA) following the manufacterer's protocol. We performed two rounds of different PCRs using three assays: (i) primer pair 42F (Oidtmann et al., 2004) and 640R (Oidtmann et al., 2006), which amplify the nuclear ribosomal internal transcribed spacer (nrITS), to test for the presence of *A. astaci*; and primers (ii) SSU (mtDNA rrnS region) and (iii) LSU (mtDNA rrnL region), to assess the intraspecific genetic diversity of *A. astaci* (Makkonen et al., 2018) and further confirm the presence of *A. astaci* (specifically with the rrnS region; Casabella-Herrero et al., 2021).

For each PCR reaction performed from cuticle extracts, the reaction mixture contained 10x Buffer, 2,5mM dNTP, 50mM $MgCl_2$, 1 mg/mL BSA, 10 mM forward and reverse primers, 5u/mL Taq-Polymerase,

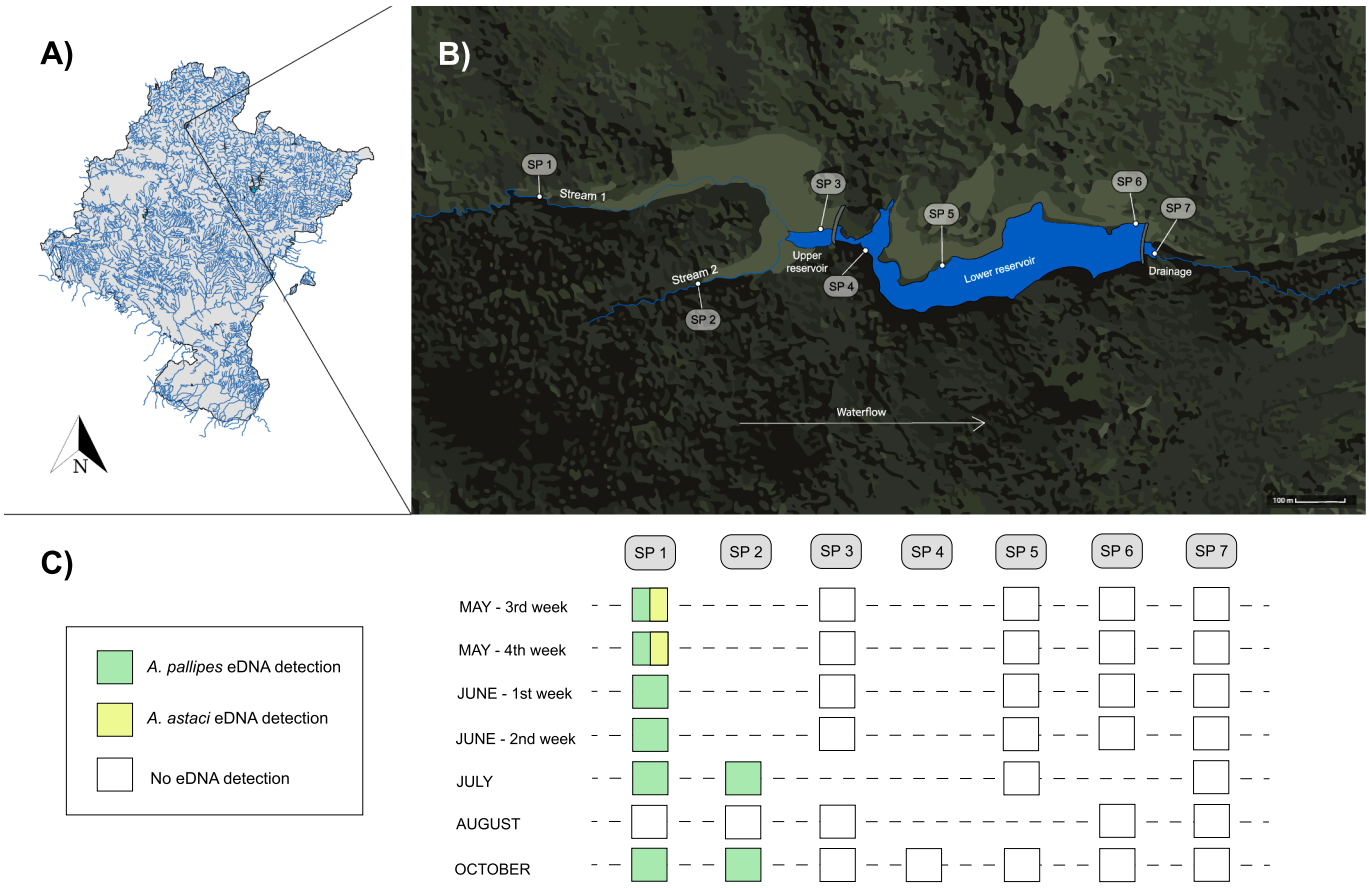

Fig. 1. Schematic representation of the area of study, sampling points and environmental DNA results (eDNA): A) map of Navarra, Spain, and the location of the Leurtza reservoirs; B) eDNA sampling points (SP) for water monitoring: SP1 – Stream 1, SP2 – Stream 2, SP3 – Upper reservoir, SP4 – Upper part of the lower reservoir, SP5 – Middle part of the lower reservoir, SP6 – Lower part of the lower reservoir, and SP7 – Drainage of the lower reservoir; C) eDNA results according to time and point of sampling: green square indicates *A. pallipes* detection; yellow square, *A. astaci* detection; white square, no eDNA detection; and no square, area not sampled. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

milliQ water, and 3 μL of extracted DNA. We also included a positive control (DNA from *A. astaci* strain CCRJB_70) and a negative control (milli-Q distilled water) per PCR reaction. PCR amplifications were checked on a 1 % agarose TAE gel stained with SYBR Safe (Thermo Fisher Scientific, Waltham, MA, USA). For the rrnS and rrnL regions, the amplified products were purified using the QIAquick PCR Purification Kit (QIAGEN) and sequenced using an automated sequencer (Applied Biosystems 3730xl DNA, Macrogen, Madrid). The obtained sequences were visualized, edited and aligned using Geneious 11.0 (Kearse et al., 2012) according to Makkonen et al. (2018). We compared the obtained sequences against a previously generated oomycete database (Casabella-Herrero et al., 2021), which included the reference haplotype-sequences of the six haplotypes detailed by Makkonen et al. (2018) and Martín-Torrijos (2018) for both mitochondrial regions (rrnS and rrnL).

2.2.2. Phylogenetic analyses and haplotyping

To characterize the genetic diversity of *A. astaci*, we performed two phylogenetic analyses, Bayesian inference (BI) and maximum likelihood (ML), with the concatenated mtDNA rrnS and rrnL regions according to Makkonen et al. (2018) and we applied the GTR+I+G model of evolution. The BI analysis was performed in Mr Bayes v.3.2.6 (Ronquist et al., 2012) for 10 million generations using the MCMC method, two runs (4 chains each), and a standard deviation of split frequencies < 0.01. We performed the ML analysis in RAxML v.8 (Stamatakis, 2014), as implemented in raxmlGUI v1.5b1 (Silvestro and Michalak, 2012), which consisted of 1000 independent replicates and 10.000 rapid bootstraps. Nodes with posterior probabilities (pp) greater than 0.95 for the BI (pp > 0.95), and greater than 75 for bootstrap values (bs) for ML (bs > 75) were considered well supported. The resulting trees were visualized and edited in FigTree v1.4.2 (Rambaut, 2012).

To estimate the most parsimonious haplotype network of *A. astaci*, we used TCS v.1.21 (Clement et al., 2002) with the obtained sequences of the rrnS+rrnL regions. Haplotype relationships were visualized using PopArt v1.7.2 (Leigh and Bryant, 2015).

2.3. eDNA procedures

2.3.1. eDNA sampling

We carried out nine sampling campaigns from May to October 2022 at seven sampling points (Fig. 1B). In each campaign, we sampled water at different sampling points according to circumstances (Fig. 1C). Water samples were collected from the upstream to downstream direction to avoid spore transport from the lower reservoir, where the crayfish mortality took place. For the filtration process, we used glass fiber filters (47 mm, 2 μm pore size, AP2504700 Millipore, MA, USA) placed inside in-line filter holders (47 mm, Millipore). Water was pumped into platinum-cured silicone tubing (Masterflex L/S, Cole-Parner, USA) using a peristaltic pump (Masterflex E/S 230 VAC, Cole-Parner, USA). After collecting the water, each filter was transferred to a sterile 15 mL Falcon tube and stored on ice until transported to the laboratory with a maximum 8 h from collection, where they were immediately stored at −20 °C. Sampling materials were thoroughly decontaminated using 10

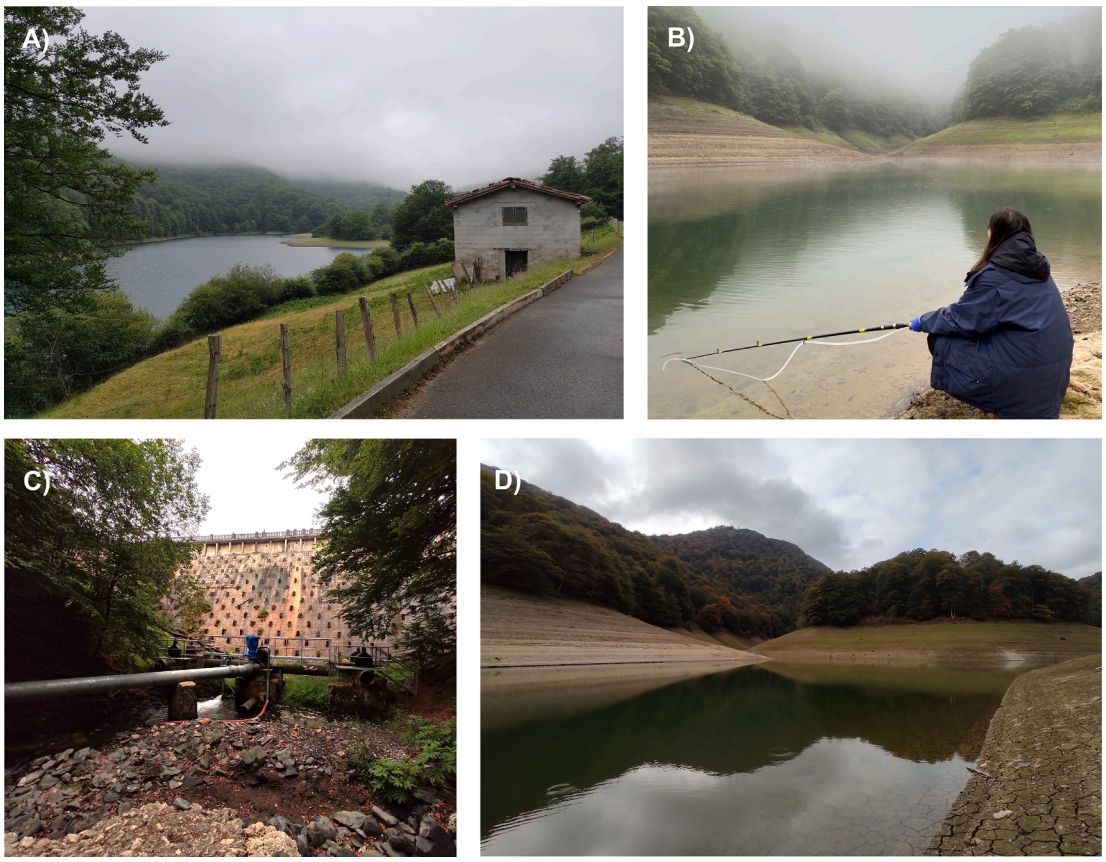

Fig. 2. Photographs of some of the Leurtza sampling points. (A) Lower reservoir at sampling point 6 (SP6), (B) Upper reservoir sampling with tubing held by a fishing rod at sampling point 3 (SP3), (C) Drainage of the lower reservoir at sampling point 6 (SP6), and (D) Upper reservoir at sampling point 3 (SP3).

% bleach for 15 min between sampling days, and different equipment (filter holder and tubing) was used between sampling points to avoid cross contamination. We aimed to filter 5 L/per site, but sometimes it was not possible due to filter clogging. To try to avoid turbidity and premature filter collapse, we sampled water at some distance from the edge of the wall (>1m) using a fishing rod fitted with the tubing (Fig. 2B), to prevent the equipment from touching the ground (Strand et al., 2019). We filtered between 1L and 5L per sample, depending on the turbidity of the water. The volume of filtered water was registered and discharged at each sampling point. A total of 91 water samples were collected, with two field replicates per sampling point; in the case of very early filter collapse (when only 1 to 2.5L of water had been filtered), we collected a third replicate.

2.3.2. eDNA extraction and qPCR analysis

The CTAB protocol described by Strand et al. (2019) was used to extract DNA from all the collected environmental samples. The extractions also included two negatives controls: (i) a tube with CTAB buffer to check for contamination during the process, and (ii) an open tube, to check for air contamination. DNA concentration of the samples was checked on a QuBit 3 (Invitrogen).

We analyzed the samples using quantitative PCR (qPCR) on an external server maintained by the Fundación Parque Científico de Madrid (Spain). We used the following four primer pairs: (i) WC2302-F/R for *A. pallipes* (Troth et al., 2020), (ii) Apall-F/R for *A. pallipes* (Atkinson et al., 2019), (iii) Paclen_COI_F0336/R0397 for *P. leniusculus* (Agersnap et al., 2017) and (iv) AphastITS-39F/97R for *A. astaci* (Vrålstad et al., 2009, with modifications by Strand et al. (2014). In the case of *A. pallipes* detection, we used two sets of primers to evaluate detection performance. For each assay, we established four calibration points in order to extrapolate DNA concentrations. For that purpose, we performed a series of 1:10 standard dilutions for each species using DNA extracted from either crayfish tissue or *A. astaci* mycelium. Three technical replicates were performed per sample, with each run containing a positive control and a non-template control (NTC) to check for contamination during the qPCR preparation procedures. For a detection to be considered positive, at least two of the three technical replicates had to contain amplifiable DNA and differences among cycle threshold (Ct) values had to be below 0.5. If the Ct of one of the replicates differed by more than 0.5 from the other, that replicate was discarded from the study (Atkinson et al., 2019). Results with a Ct greater than 40 ($Ct > 40$) were considered non-detection (Agersnap et al., 2017).

2.4. Crayfish trapping and reintroduction approach

In addition to the eDNA water sampling, we employed traditional cage traps to capture any surviving crayfish that could still be present in the water, especially in the lower reservoir. We placed three cages close to SP4, SP5, and SP6 in the lower reservoir, and one cage close to SP3 in the upper one (Fig. 1). No cages were placed in the streams or drainage due to their narrow water channel. Cages were placed at least once a week during the first month (late May-mid June) of the study and once a month for the duration of the study. The cages were left overnight with abundant bait and were checked 24 h after the eDNA sampling to avoid disturbing the sediment. In total, we placed 36 traps between May and October.

In October, we performed two more trapping experiments to make sure that the reservoir water was *A. astaci*-free before proceeding with any reintroduction program. If a single crayfish was trapped, we would not be able to reintroduce *A. pallipes* because *A. astaci* might still be present in the environment. In Spain, late October and early November are the optimal months for catching crayfish. Thus, we performed two

additional trapping experiments during this period to increase reliability of results. First, we thoroughly checked for the absence of crayfish to further confirm the eDNA results by placing 60 cages with abundant bait in the two reservoirs, 40 in the lower and 20 in the upper. The cages were left overnight and were collected after 24 h. If no crayfish were caught in the cages, we proceeded with the second test to evaluate the feasibility of reintroduction. In this experiment, we introduced cage experiments into the lower reservoir as in Taugbøl et al. (1993), *i.e.*, closed cages containing five *A. pallipes* individuals per cage and abundant bait. After a month, we checked the cages to see if crayfish were still alive. If crayfish were dead, we would test them for signs of *A. astaci* infection. However, if crayfish were still alive, we could start the reintroduction program by restocking with free-living crayfish from known populations with the same genetic diversity as the previous population.

3. Results

3.1. Crayfish samples

We observed abundant non-melanized hyphae characteristic of those from *Aphanomyces* as described by Diéguez-Uribeondo et al. (2004) in all of the examined sub-abdominal cuticles collected from the crayfish individuals that died during the mass mortality event (Fig. 3). We confirmed the presence of DNA specific to *A. astaci* via PCR (using diagnostic primers 42F and 640R) in all of the examined samples. We also identified the haplotype of the pathogen as the b-haplotype by sequencing and analyzing the concatenated sequences of the mtDNA rrnS+rrnL regions.

3.2. eDNA analyses

We detected eDNA of *A. astaci* only in Stream 1 during a two-week period in May (SP1, Fig. 1C). We detected eDNA of *A. pallipes* from May to October in Streams 1 and 2 (SP1 and SP2, respectively; Fig. 1C) at all sampling times, except August. The August samples were discarded due to signs of contamination in the extraction negative controls. Overall, the two sets of primers used to detect DNA of *A. pallipes* (Apall and WC2302) performed equally well, except in July. Both exhibited a similar level of amplification in the two field replicates per sample in most qPCR runs. For runs in which one set was weak, the other worked well. A difference in the primer performance was only observed in the analyses of the samples collected in July: although the Apall primers amplified *A. pallipes*, the WC2302 primers failed to amplify any product. Additionally, we did not detect DNA of *P. leniusculus*, *A. pallipes*, or *A. astaci* in any of the reservoirs during our sampling (Fig. 1C). We also did not detect any DNA of *P. leniusculus* from any of the sampling areas in the six-month period covering this study.

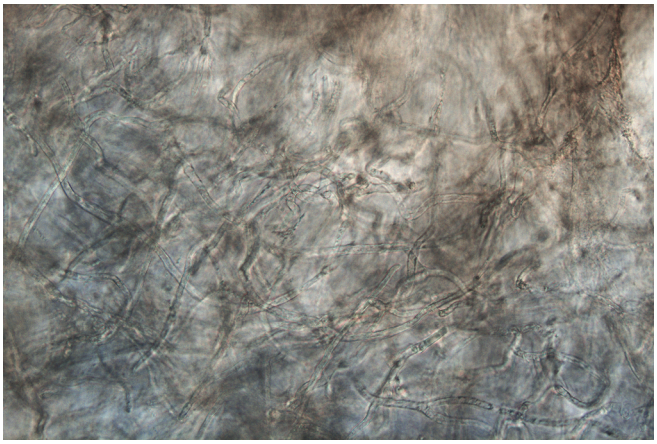

Fig. 3. Microscopic visualization of hyphae of the crayfish plague organism, *Aphanomyces astaci* colonizing the cuticle of affected *Austropotamobius pallipes* individuals in Leurtza reservoirs.

3.3. Crayfish reintroduction approach

To test for possible reintroduction of the species to Leurtza reservoirs, we first assessed whether living crayfish were still present in this locality. In the first instance, we placed a total of 36 traps over a six-month period. During the same period, we also carried out an eDNA monitoring. In the second instance, we conducted an intensive crayfish trapping experiment, placing 60 cages along the shorelines of the upper and lower reservoirs (0.5 km and 1 km of shoreline, respectively) in late October. We did not catch any crayfish using traditional trapping methods at any time point or location over the duration of these experiments. Because no crayfish were trapped during either of these trapping experiments, suggesting that the local population has become extinct, we proceeded with the second test (*i.e.*, cage experiments). It was necessary to further test the possibility of *A. astaci* or its carriers being present in the environment since non-detection with eDNA-based methods or trap-based sampling does not guarantee its absence. All crayfish kept in the introduced cages survived the surveillance period. Moreover, the results of the eDNA analyses further suggested the absence of *A. astaci* and any crayfish in the reservoirs. Therefore, we proceeded to restock the lower reservoir with 500 free-living crayfish belonging to the same genetic group as the previous population.

4. Discussion

In this study, we demonstrated that a recent mass mortality in one of the most important European populations of *A. pallipes* was caused by the pathogen *A. astaci* that originated from the IAS *P. leniusculus*. According to our analysis of the mtDNA rrnS+rrnL regions of affected crayfish, the b-haplotype was responsible for the outbreak. This haplotype has so far only been found in individuals of *P. leniusculus* or in native European crayfish infected with the crayfish plague that inhabit in the vicinity of this IAS (Huang et al., 1994, Lilley et al., 1997, Vennerström et al., 1998, Diéguez-Uribeondo and Söderhäll, 1999, Oidtmann et al., 1999, Viljamaa-Dirks et al., 2013, Makkonen et al., 2018, Martín-Torrijos et al., 2019). Widespread populations of *P. leniusculus* are known to inhabit localities near the reservoirs, and as expected, those populations are carriers of the b-haplotype (Martín-Torrijos et al., 2019).

The reintroduction of stock to the wild is a crucial aspect in the conservation management of *A. pallipes*. Here, we successfully combined eDNA monitoring and traditional trap-based sampling techniques to evaluate the reintroduction viability of *A. pallipes* in its previous habitat at Leurtza. Long-term monitoring appeared to indicate that none of the *A. pallipes* individuals survived the pathogenic invasion of the reservoirs, and that *A. astaci* and *P. leniusculus* did not remain in that freshwater environment. By analyzing eDNA, we did not detect *A. astaci* or any crayfish species (including *P. leniusculus*) in the reservoirs, nor catch any IAS or native crayfish using baited traps. However, using eDNA, we did detect the presence of *A. pallipes* at two other sampling points, Streams 1 and 2, over the entire duration of the sampling period. After careful inspection in these areas, we found no physical evidence of *A. pallipes* in the two streams, though it is plausible that a few individuals found refugia in the streams. Regarding the pathogen, we detected eDNA of *A. astaci* only in Stream 1 and only during the first two weeks of sampling. The fact that we: (i) detected the pathogen for only two weeks, (ii) did not find any sign of presence of *P. leniusculus*, and (iii) detected *A. pallipes* consistently over the six-month sampling period, seems to indicate that the crayfish inhabiting the streams were not any longer infected with crayfish plague, and that they should not be an obstacle for the restoration of the native population in the reservoirs. However, a non-detection of a species (either by eDNA monitoring or by trapping) does not necessarily imply its absence in the environment. A non-

detection of a species can be caused by several reasons: (i) sampling method bias, (ii) rapid eDNA degradation, or (iii) low eDNA concentration, that makes eDNA sampled to be below the limit of detection for a qPCR assay. Similarly, for trap-based sampling, traps cannot attract all specimens in a population due to the size or positioning along the waterbodies. For these reasons, and because the reservoirs seemed to be free of *A. astaci* at the end of the monitoring period, we performed two additional reintroduction experiments (intensive trap-based sampling and cage experiments) prior to the start of the crayfish reproductive season (late October to early November in Spain) to further confirm our assumptions that the reservoir was free of *A. astaci* and *P. leniusculus*. Specifically, cage experiments were successfully used for decades to evaluate the viability of the reintroduction of susceptible crayfish (Taugbøl et al., 1993, Diéguez-Uribeondo et al., 1997b; Spink and Frayling, 2000). Overall, the results of our experiments support the absence of invasive crayfish and pathogen in the reservoirs. Thus, we concluded that a reintroduction plan was viable for this location, and we reintroduced 500 adult individuals in the lower reservoir. Continued monitoring of this population over the next three to five years will be used to assess the success of this restocking program and the stability of the population. Nightingale et al., (2017) reviewed crayfish reintroductions and introduction studies conducted in the UK and Ireland and concluded that only 26 of 59 cases had been successful. However, successful reintroductions after crayfish plague events have been reported from several countries including Norway, Italy, and the UK (Taugbøl et al., 1993, Spink and Frayling, 2000, Manenti et al., 2021), as well as in other regions of Navarra in Spain (Diéguez-Uribeondo et al., 1997b).

Compared with traditional trap-based sampling, the eDNA monitoring method was more efficient in our study because it required less time-investment and effort for sampling, and provided more and faster information. Based on the results of this sampling method, we hypothesize three possible entrance points of *A. astaci* to the Leurtza water system, and to Stream 1 that resulted in the crayfish plague outbreak that decimated the native population: (i) in the lower reservoir, (ii) in the upper reservoir, or (iii) in Stream 1. In the first scenario, *A. astaci* was first introduced to the lower reservoir, and owing to the high density of crayfish there, the infection spread rapidly. Then, some infected *A. pallipes* might have migrated to the upper reservoir, and then to the Stream 1. In the second scenario, in which the pathogen was introduced first to the upper reservoir, we would have to assume that some crayfish were not only present there, but were infected. The outbreak occurred in May, when water connection between reservoirs is frequent; this would have favored *A. astaci* reaching the downstream crayfish population. As in the first scenario, infected crayfish would have then migrated to Stream 1, which could explain the detection of *A. astaci* eDNA in that area in the weeks after the outbreak. In the third scenario, *A. astaci* was introduced to the Stream 1, and would have followed the watercourse to the lower reservoir, where it caused the outbreak. In this scenario, we would have expected to detect eDNA of *A. astaci* and/or *A. pallipes* in the lower reservoir during the first weeks of sampling, and not exclusively in Stream 1. Previous studies have reported increased detection of *A. astaci* and susceptible crayfish during and after outbreaks of the pathogen (Strand et al., 2014, 2019). However, by reconstructing the timeline of the outbreak, we estimated that the infection process could have started two to three weeks before our sampling. This period is long enough to allow the local extinction of the crayfish population in the lower reservoir, and the rapid degradation of eDNA of both pathogen and host, with the few surviving individuals of *A. pallipes* in Stream 1 favoring a longer presence of *A. astaci* in that stream. In addition, the streams originate from underground watercourses with much colder temperatures, which might have slowed the degradation of eDNA in these streams compared with than in the reservoir waters (*e.g.*, Barnes et al., 2014, Strickler et al., 2015). Another possibility is that the detection of *A. astaci* in the Stream 1 was a false positive due to a potential presence of a closely related *Aphanomyces* species (Viljamaa-Dirks and Heinikainen, 2019).

Overall, our study demonstrates that eDNA sampling is an informative tool for evaluating reintroduction plans, since it allows monitoring of crayfish and the pathogen, and facilitates the decision-making process in the management of endangered native species and its pathogen. Although our methodology did not strictly follow the existing criteria of reintroduction for crayfish described by Souty-Grosset and Reynolds (2009), we have followed the protocol described by the official plan of conservation of *A. pallipes* in Navarra, which so far has been successful (Diéguez-Uribeondo et al., 1997b). We encourage future studies to complement traditional detection methods with eDNA monitoring (Sepulveda et al., 2020, King et al., 2022) in the detection of crayfish and *A. astaci*. Our study is yet another example that highlights that *A. astaci* still poses a very severe threat to susceptible native crayfish populations after more than 50 years of documented presence in the Iberian Peninsula. Its continuing threat to native populations emphasizes the need for increased prevention measures against IAS crayfish translocations, greater awareness among the public (Manenti et al., 2021) and monitoring of key or high-risk infection areas to avoid further *A. astaci* expansion.

5. Conclusions

1. eDNA is a valuable tool for field detection of emerging diseases, and native species in freshwater environments.
2. The combination of eDNA together with traditional trap-based methods, can be applied in conservation management, *e.g.*, evaluation of reintroduction strategies.
3. eDNA monitoring can help accelerate effective conservation decision-making.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

This work was supported by the Government of Navarra, Spain. We would like to acknowledge María Eugenia Hernando Pascual, Jokin Larumbe Arricibita and Guarderio de Medio Ambiente of the Government of Navarra for supporting this project. Gloria Casabella-Herrero was supported by the project IND2019/AMB-17177, Consejería de Ciencia y Universidades de Innovación, Comunidad de Madrid, Spain, and Langenomics Biodetect, Spain SL. Drs. Laura Martín-Torrijos and Javier Diéguez Uribeondo were supported by project of the Spanish Ministry of Science and Innovation (I+D+I) PID2020-16115GB-100.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jip.2023.108026.

References

Agersnap, S., Larsen, W.B., Knudsen, S.W., Strand, D., Thomsen, P.F., Hesselsoe, M., Mortensen, P.B., Vrålstad, T., Moller, P.R., 2017. Monitoring of noble, signal and narrow-clawed crayfish using environmental DNA from freshwater samples. PLoS One 12 (6), e0179261. https://doi.org/10.1371/journal.pone.0179261.

Alonso, F., Temiño, C., Diéguez-Uribeondo, J., 2000. Status of the white-clawed crayfish, *Austropotamobius pallipes* (Lereboullet, 1858), in Spain: distribution and legislation. Bulletin Français De La Pêche Et De La Pisciculture 356, 31–53. https://doi.org/10.1051/kmae:2000003.

Atkinson, S., Carlsson, J.E.L., Ball, B., Kelly-Quinn, M., Carlsson, J., 2019. Field application of an eDNA assay for the threatened white-clawed crayfish *Austropotamobius pallipes*. Freshwater Science 38 (3), 503–509. https://doi.org/10.1086/704712.

Barnes, M.A., Turner, C.R., Jerde, C.L., Renshaw, M.A., Chadderton, W.L., Lodge, D.M., 2014. Environmental conditions influence eDNA persistence in aquatic systems. Environ. Sci. Technol. 48 (3), 1819–1827. https://doi.org/10.1021/es404734p.

Casabella-Herrero, G., Martínez-Ríos, M., Viljamaa-Dirks, S., Martín-Torrijos, L., Diéguez-Uribeondo, J., 2021. *Aphanomyces astaci* mtDNA: insights into the pathogen's differentiation and its genetic diversity from other closely related oomycetes. Fungal Biol. 125 (4), 316–325. https://doi.org/10.1016/j.funbio.2020.11.010.

Cheslett, D., Geary, M., Brenan, A., Viljamaa-Dirks, S., Panteleit, J., 2019. The National Crayfish Plague Surveillance Program, Ireland-2018-2019.

Chucholl, F., Fiolka, F., Segelbacher, G., Epp, L.S., 2021. eDNA detection of native and invasive crayfish species allows for year-round monitoring and large-scale screening of lotic systems. Front. Environ. Sci. 9, 639380 https://doi.org/10.3389/fenvs.2021.639380.

Clement, M., Snell, Q., Walker, P., Posada, D., Crandall, K., 2002. TCS: estimating gene genealogies. In Parallel and Distributed Processing Symposium. International. IEEE Computer Society 3, 0184.

Conn, D.B., 2014. Aquatic invasive species and emerging infectious disease threats: A One Health perspective. Aquatic Invasions 9 (3), 383–390. https://doi.org/10.3391/ai.2014.9.3.12.

Diéguez-Uribeondo, J., Temiño, C., Múzquiz, J.L., 1997a. The crayfish plague fungus (*Aphanomyces astaci*) in Spain. Bulletin Français De Pêche Piscicole 347, 753–763. https://doi.org/10.1051/kmae/1997051.

Diéguez-Uribeondo, J., Rueda, A., Castien, E., Bascones, J.C., 1997b. A plan of restauration in Navarra for the native freshwater crayfish species of Spain, *Austropotamobius pallipes*. Bulletin Français De La Pêche Et De La Pisciculture 347, 625–637. https://doi.org/10.1051/kmae/1997056.

Diéguez-Uribeondo, J., Söderhäll, K., 1999. RAPD evidence for the origin of an outbreak of crayfish plague in Spain. Freshwater Crayfish 12, 313–318.

Diéguez-Uribeondo, J., Gierz, G., Bartnicki-Garcia, S., 2004. Image analysis of hyphal morphogenesis in Saprolegniaceae (Oomycetes). Fungal Genet. Biol. 41 (3), 293–307. https://doi.org/10.1016/j.fgb.2003.10.012.

Fisher, M.C., Henk, D.A., Briggs, C.J., Brownstein, J.S., McCraw, S.L., Gurr, S.J., 2012. Emerging fungal threats to animal, plants and ecosystem health. Nature 484, 186–194. https://doi.org/10.1038/nature10947.

Goldberg, C.S., Turner, C.R., Deiner, K., Klymus, K.E., Thomsen, P.F., Murphy, M.A., Spear, S.F., McKee, A., Oyler-McCance, S.J., Cornman, R.S., Laramie, M.B., Mahon, A.R., Lance, R.F., Pilliod, D.S., Strickler, K.M., Waits, L.P., Fremier, A.K., Takahara, T., Herder, J.E., Taberlet, P., 2016. Critical considerations for the application of environmental DNA methods to detect aquatic species. Methods Ecol. Evol. 7 (11), 1299–1307. https://doi.org/10.1111/2041-210X.12595.

Gomes, G.B., Hutson, K.S., Domingos, J.A., Chung, C., Hayward, S., Miller, T.L., Jerry, D. R., 2017. Use of environmental DNA (eDNA) and water quality data to predict protozoan parasites outbreaks in fish farms. Aquaculture 479, 467–473. https://doi.org/10.1016/j.aquaculture.2017.06.021.

Grandjean, F., Vrålstad, T., Diéguez-Uribeondo, J., Jelić, M., Mangombi, J., Delaunay, C., Filipová, L., Rezinciuc, S., Kozubíková-Balcarová, E., Guyonnet, D., Viljamaa-Dirks, S., Petrusek, A., 2014. Microsatellite markers for direct genotyping of the crayfish plague pathogen *Aphanomyces astaci* (Oomycetes) from infected host tissues. Vet. Microbiol. 170 (3–4), 317–324. https://doi.org/10.1016/j.vetmic.2014.02.020.

Habsburgo-Lorena, A.S., 1979. Present situation of exotic species of crayfish introduced into Spanish continental waters. Freshwater Crayfish 4, 175–184.

Havel, J.E., Kovalenko, K.E., Thomaz, S.M., Amalfitano, S., Kats, L.B., 2015. Aquatic invasive species: challenges for the future. Hydrobiologia 750, 147–170. https://doi.org/10.1007/s10750-014-2166-0.

Huang, T.S., Cerenius, L., Söderhäll, K., 1994. Analysis of genetic diversity in the crayfish plague fungus, *Aphanomyces astaci*, by random amplification of polymorphic DNA. Aquaculture 126 (1–2), 1–9. https://doi.org/10.1016/0044-8486(94)90243-7.

Jussila, J., Edsman, L., Maguire, I., Diéguez-Uribeondo, J., Theissinger, K., 2021. Money kills native ecosystems: European crayfish as an example. Front. Ecol. Evol. 9, 648495 https://doi.org/10.3389/fevo.2021.648495.

Kearse, M., Moir, R., Wilson, A., Stones-Havas, S., Cheung, M., Sturrock, S., Buxton, S., Cooper, A., Markowitz, S., Duran, C., Thierer, T., Ashton, B., Meintjes, P., Drummond, A., 2012. Geneious Basic: an integrated and extendable desktop software platform for the organization and analysis of sequence data. Bioinformatics 28 (12), 1647–1649. https://doi.org/10.1093/bioinformatics/bts199.

King, A.C., Krieg, R., Weston, A., Zenker, A.K., 2022. Using eDNA to simultaneously detect the distribution of native and invasive crayfish within an entire country. J. Environ. Manage. 302, 113929 https://doi.org/10.1016/j.jenvman.2021.113929.

Larson, E.R., Graham, M., Achury, R., Coon, J.J., Gambrell, D.K., Jonasen, K.L., King, G. D., LaRacuente, A., Perrin-Stowe, T.I.N., Reed, E.M., Rice, C.J., Ruzei, S.A., Thairu, M.W., Wilson, J.C., Suarez, A.V., 2020. From eDNA to citizen science: emerging tools for the early detection of invasive species. Front. Ecol. Environ. 18 (4), 194–202. https://doi.org/10.1002/fee.2162.

Leigh, J.W., Bryant, D., 2015. POPART: full-feature software for haplotype network construction. Methods Ecol. Evol. 6 (9), 1110–1116. https://doi.org/10.1111/2041-210x.12410.

Lilley, J.H., Cerenius, L., Söderhäll, K., 1997. RAPD evidence for the origin of the crayfish plague outbreaks in Britain. Aquaculture 157, 181–185. https://doi.org/10.1016/S0044-8486(97)00153-1.

Lowe, S., Browne, M., Buoudjelas, S., De Poorter, M., 2004. 100 of the world's worst invasive alien species: a selection from the global invasive species database The invasive species specialist group (ISSG), a specialist group of the species survival commission (SSC) of the IUCN 2004 Auckland, New Zealand Accessed in http://www.issg.org/pdf/publications/worst_100/english_100_worst.pdf.

Makkonen, J., Jussila, J., Kokko, H., 2012. The diversity of the pathogenic Oomycete (*Aphanomyces astaci)* chitinase genes within the genotypes indicate adaptation to its hosts. Fungal Genet. Biol. 49 (8), 635–642. https://doi.org/10.1016/j.fgb.2012.05.014.

Makkonen, J., Jussila, J., Panteleit, J., Keller, N.S., Schrimpf, A., Thessinger, K., Kortet, R., Martín-Torrijos, L., Diéguez-Uribeondo, J., Kokko, H., 2018. MtDNA allows the sensitive detection and haplotyping of the crayfish plague disease agent *Aphanomyces astaci* showing clues about its origin and migration. Parasitology 145 (9), 1210–1218. https://doi.org/10.1017/S0031182018000227.

Manenti, R., Barzaghi, B., Nessi, A., Cioccarelli, S., Villa, M., Ficetola, G.F., 2021. Not only environmental conditions but also human awareness matters: a successful post-crayfish plague reintroduction of the white-clawed crayfish (*Austropotamobius pallipes*) in Northern Italy. Front. Ecol. Evol. 9, 621613 https://doi.org/10.3389/fevo.2021.621613.

Martín-Torrijos, L., Kawai, T., Makkonen, J., Jussila, J., Kokko, H., Diéguez-Uribeondo, J., 2018. Crayfish plague in Japan: A real threat to the endemic *Cambaroides japonicus*. PLoS One 13 (4), e0195353. https://doi.org/10.1371/journal.pone.0195353.

Martín-Torrijos, L., Kokko, H., Makkonen, J., Jussila, J., Diéguez-Uribeondo, J., 2019. Mapping 15 years of the crayfish plague in the Iberian Peninsula: The impact of two invasive species on the endangered native crayfish. PLoS One 14 (8), e0219223. https://doi.org/10.1371/journal.pone.0219223.

Martín-Torrijos, L., Martínez-Ríos, M., Casabella-Herrero, G., Adams, S.B., Jackson, C., Diéguez-Uribeondo, J., 2021. Tracing origin of the crayfish plague pathogen, *Aphanomyces astaci*, to the Southeastern United States. Sci. Rep. 11, 9332. https://doi.org/10.1038/s41598-021-88704-8.

Martín-Torrijos, L., Hernández-Pérez, A., Monroy-López, J.F., Diéguez-Uribeondo, J., 2023. *Aphanomyces astaci* in Mexico: A new haplotype from dwarf crayfish *Cambarellus montezumae*. J. Invertebr. Pathol. 201, 108000 https://doi.org/10.1016/j.jip.2023.108000.

Morisette, J., Burgiel, S., Brantley, K., Daniel, W.M., Darling, J., Davis, J., Franklin, T., Gaddis, K., Hunter, M., Lance, R., Leskey, T., Passamaneck, Y., Piaggio, A., Rector, B., Sepulveda, A., Smith, M., Nightingale, J., Stebbing, P., Sibley, P., Brown, O., Rushbrook, B., Jones, G., 2017. A review of the use of ark sites and associated conservation measures to secure the long-term survival of white-clawed crayfish *Austropotamobius pallipes* in the United Kingdom and Ireland. International Zoo Yearbook 51 (1), 50–68. https://doi.org/10.1111/izy.12161.

Oidtmann, B., Cerenius, L., Schmid, I., Hoffmann, R., Söderhäll, K., 1999. Crayfish plague epizootics in Germany - classification of two German isolates of the crayfish plague fungus *Aphanomyces astaci* by random amplification of polymorphic DNA. Dis. Aquat. Organ. 35 (3), 235–238. https://doi.org/10.3354/dao035235.

Oidtmann, B., Schaefers, N., Cerenius, L., Söderhäll, K., Hoffmann, R.W., 2004. Detection of genomic DNA of the crayfish plague fungus *Aphanomyces astaci* (Oomycete) in clinical samples by PCR. Veterinary Microbiology 100 (3–4), 269–282. https://doi.org/10.1016/j.vetmic.2004.01.019.

Oidtmann, B., Geiger, S., Steinbauer, P., Culas, A., Hoffmann, R.W., 2006. Detection of *Aphanomyces astaci* in North American crayfish by polymerase chain reaction. Dis. Aquat. Organ. 72 (1), 53–64. https://doi.org/10.3354/dao072053.

Rambaut, A., 2012. Figtree v1.4.0. Accessed in http://tree.bio.ed.ac.uk/software/figtree/.

Rees, H.C., Maddison, B.C., Middleditch, D.J., Patmore, J.R.M., Gough, K.C., 2014. The detection of aquatic animal species using environmental DNA – a review of eDNA as a survey tool in ecology. J. Appl. Ecol. 51, 1450–1459. https://doi.org/10.1111/1365-2664.12306.

Reid, A.J., Carlson, A.K., Creed, I.F., Eliason, E.J., Gell, P.A., Johnson, P.T., Kidd, K.A., MacCormack, T.J., Olden, J.D., Ormerod, S.J., Smol, J.P., Taylor, W.W., Tockner, K., Vermaire, J.C., Dudgeon, D., Cooke, S.J., 2019. Emerging threats and persistent conservation challenges for freshwater biodiversity. Biol. Rev. 94 (3), 849–873. https://doi.org/10.1111/brv.12480.

Rezinciuc, S., Sandoval-Sierra, J.V., Oidtmann, B., Diéguez-Uribeondo, J., 2015. The Biology of Crayfish Plague Pathogen *Aphanomyces astaci:* Current Answers to Most Frequent Questions. In: Kawai, T., Faulkes, Z., Scholtz, G. (Eds.), Freshwater Crayfish. CRC Press, San Diego (USA), pp. 182–204.

Ronquist, F., Teslenko, M., van der Mark, P., Ayres, D.L., Darling, A., Hohna, S., Larget, B., Liu, L., Suchard, M., Huelsenbeck, J.P., 2012. MrBayes 3.2: efficient Bayesian phylogenetic inference and model choice across a large model space. Syst. Biol. 61 (3), 539–542. https://doi.org/10.1093/sysbio/sys029.

Rusch, J.C., Mojžišová, M., Strand, D.A., Svobodová, J., Vrålstad, T., Petrusek, A., 2020. Simultaneous detection of native and invasive crayfish and *Aphanomyces astaci* from environmental DNA samples in a wide range of habitats in Central Europe. NeoBiota 58, 1–32. https://doi.org/10.3897/neobiota.58.49358.

Sepulveda, A.J., Nelson, N.M., Jerde, C.L., Luikart, G., 2020. Are environmental DNA methods ready for aquatic invasive species management? Trends Ecol. Evol. 35 (8), 668–678. https://doi.org/10.1016/j.tree.2020.03.011.

Sieber, N., Hartikainen, H., Vorburger, C., 2020. Validation of an eDNA-based method for the detection of wildlife pathogens in water. Dis. Aquat. Organ. 141, 171–184. https://doi.org/10.3354/dao03524.

Sigsgaard, E.E., Carl, H., Moller, P.R., Thomsen, P.F., 2015. Monitoring the near-extinct European weather loach in Denmark based on environmental DNA from water samples. Biol. Conserv. 183, 46–52. https://doi.org/10.1016/j.biocon.2014.11.023.

Silvestro, D., Michalak, I., 2012. raxmlGUI: a graphical front-end for RAxML. Organisms Diversity Evol. 12, 355–357.

Souty-Grosset, C., Reynolds, J.D., 2009. Current ideas on methodological approaches in European crayfish conservation and restocking procedures. Knowl. Manag. Aquat. Ecosyst. 01, 394–395.

Spink, J.M., Frayling, M.W., 2000. An assessment of post-plague reintroduced native white-clawed crayfish *Austropotamobius pallipes*, in the Sherston Avon and Tetbury Avon, Wiltshire. Freshwater Forum 14, 59–67.

Stamatakis, A., 2014. RAxML version 8: a tool for phylogenetic analysis and post analysis of large phylogenies. Bioinformatics 30 (9), 1312–1313. https://doi.org/10.1093/bioinformatics/btu033.

Strand, D.A., Jussila, J., Viljamaa-Dirks, S., Kokko, H., Makkonen, J., Holst-Jensen, A., Viljugrein, H., Vrålstad, T., 2012. Monitoring the spore dynamics of *Aphanomyces astaci* in the ambient water of latent carrier crayfish. Vet. Microbiol. 160 (1–2), 99–107. https://doi.org/10.1016/j.vetmic.2012.05.008.

Strand, D.A., Jussila, J., Johnsen, S.I., Viljamaa-Dirks, S., Edsman, L., Wiik-Nielsen, J., Viljugrein, H., Engdahl, F., Vrålstad, T., 2014. Detection of crayfish plague spores in large freshwater systems. J. Appl. Ecol. 51 (2), 544–553. https://doi.org/10.1111/1365-2664.12218.

Strand, D.A., Johnsen, S.I., Rusch, J.C., Agersnap, S., Larsen, W.B., Knudsen, S.W., Møller, P.R., Vrålstad, T., 2019. Monitoring a Norwegian freshwater crayfish tragedy: eDNA snapshots of invasion, infection and extinction. J. Appl. Ecol. 56 (7), 1661–1673. https://doi.org/10.1111/1365-2664.13404.

Strickler, K.M., Fremier, A.K., Goldberg, C.S., 2015. Quantifying effects of UV-B, temperature, and pH on eDNA degradation in aquatic microcosms. Biol. Conserv. 183, 85–92. https://doi.org/10.1016/j.biocon.2014.11.038.

Taugbøl, T., Skurdal, J., Håstein, T., 1993. Crayfish plague and management strategies in Norway. Biol. Conserv. 63 (1), 75–82. https://doi.org/10.1016/0006-3207(93)90076-D.

Tréguier, A., Paillisson, J.M., Dejean, T., Valentini, A., Schlaepfer, M.A., Roussel, J.M., 2014. Environmental DNA surveillance for invertebrate species: advantages and technical limitations to detect the invasive crayfish *Procambarus clarkii* in freshwater ponds. J. Appl. Ecol. 51, 871–879. https://doi.org/10.1111/1365-2664.12262.

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

Vennerström, P., Söderhäll, K., Cerenius, L., 1998. The origin of two crayfish plague (*Aphanomyces astaci*) epizootics in Finland on noble crayfish Astacus astacus. Ann. Zool. Fenn. 35, 43–46.

Viljamaa-Dirks, S., Heinikainen, S., Torssonen, H., Pursiainen, M., Mattila, J., Pelkonen, S., 2013. Distribution and epidemiology of genotypes of the crayfish plague agent *Aphanomyces astaci* from noble crayfish *Astacus astacus* in Finland. Dis. Aquat. Organ. 103 (3), 199–208. https://doi.org/10.3354/dao02575.

Viljamaa-Dirks, S., Heinikainen, S., 2019. A tentative new species *Aphanomyces fennicus* sp. nov. interferes with molecular diagnostic methods for crayfish plague. J. Fish Dis. 42 (3), 413–422. https://doi.org/10.1111/jfd.12955.

Voyles, J., Kilpatrick, A.M., Collins, J.P., Fisher, M.C., Frick, W.F., McCallum, H., Willis, C.K.R., Blehert, D.S., Murray, K.A., Puschendorf, R., Rosenblum, E.B., Bolker, B.M., Chenf, T.L., Langwig, K.E., Lindner, D.L., Toothman, M., Wilber, M.Q., Briggs, C.J., 2015. Moving beyond too little, too late: managing emerging infectious diseases in wild populations requires international policy and partnerships. Ecohealth 12, 404–407. https://doi.org/10.1007/s10393-014-0980-5.

Vrålstad, T., Knutsen, A.K., Tengs, T., Holst-Jensen, A., 2009. A quantitative TaqMan MGB real-time polymerase chain reaction-based assay for detection of the causative agent of crayfish plague *Aphanomyces astaci*. Vet. Microbiol. 137, 146–155. https://doi.org/10.1016/j.vetmic.2008.12.022.

Wittwer, C., Stoll, S., Strand, D., Vrålstad, T., Nowak, C., Thines, M., 2018. eDNA-based crayfish plague monitoring is superior to conventional trap-based assessments in year-round detection probability. Hydrobiologia 807, 87–97. https://doi.org/10.1007/s10750-017-3408-8.

Wittwer, C., Stoll, S., Thines, M., Nowak, C., 2019. eDNA-based crayfish plague detection as practical tool for biomonitoring and risk assessment of A. astaci-positive crayfish populations. Biol. Invasions 21, 1075–1088. https://doi.org/10.1007/s10530-018-1886-x.