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ORIGINAL ARTICLE



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Influence of distance from source population and seasonality in eDNA detection of white-clawed crayfish, through qPCR and ddPCR assays

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Abstract

The white-clawed crayfish (Austropotamobius pallipes) is an emblematic taxon of European rivers, found mainly in oxygenated streams, known to be an excellent indicator of river quality. Since several decades, the population of A. pallipes declined in relation to anthropogenic pressure, habitat loss, and competition with pests (invasive crayfish, crayfish plague). This endangered species is now submitted to conservation strategies by freshwater managers in order to survey and protect the remaining populations. In France, traditional surveys in freshwater environments were performed by electric fishing, kick-net fishing, or trapping, particularly disruptive for the environment and very time-consuming. However, with the rise of molecular genetic technology, new methods based on the detection of environmental DNA (eDNA) have emerged. We present here the results of an optimized study for the detection of the endangered crayfish Austropotamobius pallipes in France, considering certain environmental co-factors and comparing two PCR methods (gPCR and ddPCR). After improving laboratory procedures, we were able to detect the presence of the crayfish up to 2km downstream from a known point of presence and unfortunately highlight the disappearance of a historical population, after sampling two consecutive years. Such a level of precision is interesting because it makes it possible to precise the presence of specimens in a relatively restricted area and to orient traditional prospecting, necessary for certain additional studies. During our study, we observed better probabilities of detection during the summer period, but in a growing context of climate change, we advise to adapt the sampling year by year. That said, this methodology is a very useful tool for the detection of rare and/or endemic species and we did not observe any difference between the two PCR methods used.

KEYWORDS

Austropotamobius pallipes, conservation, crayfish, downstream detection, droplet digital PCR, environmental DNA, quantitative PCR, seasonality

Thomas Baudry and Maud Laffitte are co-first authors.

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1 | INTRODUCTION

Biological inventories of macro-organisms in aquatic environments were initially based on traditional methods like direct capture, electrofishing, or baited traps, known to be particularly disruptive for the environment, non-selective, and time-consuming (Hänfling et al., 2016; Robinson et al., 2018; Wang et al., 2021). The development of molecular genetic methods has made it possible to apply novel strategies for aquatic biodiversity assessment using environmental DNA (eDNA; Bruce et al., 2021; Ruppert et al., 2019). As all the living organisms, from bacteria to vertebrates, shed genetic material into their surrounding environment via natural biological processes (e.g., mucus secretion, feces excretion, cells release), the application of molecular approaches to eDNA extracted from environmental samples opens a new way to study biodiversity (Barnes & Turner, 2015). The increasing use of eDNA-based approaches is such that it can be considered as a disciplinary field, and studies have been done to investigate the "ecology of eDNA" and to fully understand its potential and limitations for biodiversity assessment.

In freshwater ecosystems, the first species-specific eDNAbased approach was developed by Ficetola et al. (2008), proving its effectiveness to detect an invasive frog (Rana catesbeiana) in France. Since a large number of studies and methodological development were conducted to improve eDNA-based applications (Schenekar, 2022), in particular as an aquatic conservation tool to monitor taxa of interest (e.g., invasive, rare, threatened taxa), using eDNA collected from river water samples (e.g., Atkinson et al., 2019; Dubreuil et al., 2021; Harper et al., 2018; Piggott, 2016). However, detection and interpretation of eDNA signals from water samples in rivers can be challenging due to natural dilution, degradation (environmental factors, biological processes), and transport conditions (Barnes & Turner, 2015). To overcome those potential biases, optimization of water sampling strategies (e.g., point sampling vs. integrated sampling), field samples preservation (e.g., dry, in ethanol, on ice, in buffer, etc...), eDNA isolation (e.g., filtration vs. centrifugation) and extraction (e.g., Phenol-Chloroform vs. commercial kits) were evaluated in previous studies (e.g. Deiner et al., 2015; Djurhuus et al., 2017; Goldberg et al., 2011; Majaneva et al., 2018). There is no clear agreement of what the best strategy is, but it appears from the literature that: (i) water filtration implemented directly onsite, from an integrated sampling, seemed to provide higher yields (Deiner et al., 2015; Troth et al., 2020), (ii) filtration of large volumes of water (up to several tens of liters) allows to capture a larger number of eDNA fragments (Goldberg et al., 2016; Majaneva et al., 2018), and (iii) preservation onsite reduces its potential degradation (Carraro et al., 2021), (iv) better eDNA extraction yields (from filters) seem to be obtained with commercial DNA extraction kits (e.g. Qiagen DNeasy blood and tissue; Deiner et al., 2015; Djurhuus et al., 2017). Concerning the samples analyses, several studies have recently compared the sensitivity of two Polymerase Chain Reaction (PCR) methods, digital droplet PCR (ddPCR) and quantitative PCR (qPCR), each with their own merits (Doi et al., 2015; Mauvisseau et al., 2019). The former is known to be more sensitive (in case of low abundant

signal, or potential inhibition effect), but has higher costs and longer experimental time (Doi et al., 2015). While the latter is less accurate for eDNA quantification, but it is simple to apply, often less expensive (depending on the working context), and generates rapid results (Mauvisseau et al., 2019).

If methodological aspects of eDNA-based approaches have intensively been explored, the success of eDNA applications is also dependent on the ecology and population biology of the targeted species. Seasonal variation in activity, abundance, habitat patchiness, influencing the population-level excretion of DNA (Dunn et al., 2017; Troth et al., 2021) and the distance of water sampling points from the major source population (Deiner & Altermatt, 2014; Jane et al., 2015) will influence recoverable eDNA. In temperate zones, strong seasonal variations in temperatures and therefore metabolic rate and activity of aquatic species can be observed, influencing the eDNA detection success (Dunn et al., 2017; Troth et al., 2021). For example, eDNA concentration peaks (20 times higher) are observed during reproduction and spawning in pearl mussels (Wacker et al., 2019). A similar observation was made in crayfish (Austropotamobius pallipes and Pacifastacus leniusculus) during the spawning and molting periods (Dunn et al., 2017; Troth et al., 2021). As a consequence, an unadapted eDNA sampling strategy could lead to false negative results of the target species depending on the season, affecting both river hydrology and species activity or in sub-optimal habitats, which is particularly the case with taxa harboring complex annual life cycle like the white-clawed crayfish, A. pallipes (King et al., 2022).

White-clawed crayfish represents a model species because it is a large aquatic species inhabiting small creeks and streams and is of ecological, conservation importance and interest in Europe (Grandiean et al., 2000: Kouba et al., 2014: Préau et al., 2020). Its strict ecological requirements for pristine aquatic environments make it a valuable bioindicator species (Trouilhé et al., 2007), with its presence indicating a high-quality aquatic environment. However, it is also highly threatened in Europe with a small fragmented and generally declining population that can be challenging to detect and monitor (Füreder et al., 2010). The number of white-clawed crayfish has fallen significantly, with losses estimated between 50% and 80% since the 2000s across Europe (Collas et al., 2016; Füreder et al., 2010), due to the combined effects of habitat loss, decrease in water quality, but also competition with introduced American crayfish species (Faxonius limosus, P. leniusculus, Procambarus clarkii), carriers of the crayfish plague, Aphanomyces astaci (Becking et al., 2021; Grandjean et al., 2017). This leads to an increasingly diminished presence in France, with low A. pallipes population densities at known sites, difficult, and time-consuming to detect using traditional sampling approaches, explaining why eDNA-based approaches are developed to complement traditional surveys and improve conservation strategies.

In this study, we investigated the effect of (i) seasonality; (ii) environmental variables (temperature and oxygen); (iii) the sampling distance from a source population on the detection efficiency of eDNA methods for A.pallipes. Two molecular methods commonly applied for eDNA species-specific surveys were used (qPCR and ddPCR)

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and compared in terms of eDNA detection efficiency. Investigations were performed in 10 streams on site where known white-crayfish populations were described by direct observations and/or trapping. Our results will contribute to scientific knowledge regarding eDNA species-specific signal interpretation in rivers. We will also discuss about the pertinence of eDNA-based approach for conservation purpose in rivers and its operationality in the particular context of the white-clawed crayfish.

2 | MATERIALS AND METHODS

2.1 | Sampling sites and water sampling

This study took place in Indre et Loire, a department located in central France and crossed by the Loire River and its many tributaries (Figure 1). Traditional night crayfish surveys were carried out by local authorities (Fédération de Pêche de l'Indre et Loire) during the summer of 2021 to confirm the historical presence of *A. pallipes* at different river stations of the department using trapping and spotlighting (direct observation) (Bryant et al., 2012). According to their results, nine brooks (plus one in 2022) distributed in different water basins of the department and with an identified population of *A. pallipes* were selected for our study (see above "Section 2.2. Seasonal and spatial eDNA sampling strategies").

Water samples used to perform eDNA-based approaches were collected during two sampling campaigns realized in summer 2021 and 2022. Adjusted sampling strategies were defined to evaluate the spatial and seasonal variation of eDNA signal which are described in the section "Section 2.2. Seasonal and spatial eDNA sampling strategies." At each location, water from the stream was collected, from one bank to the other, using three independent DNA-free plastic bottles (1L each and so 3L per station, to be sure to have enough water for filtrations) and non-powdered gloves following Cowart

et al. (2018). The water samples were kept in a cooler until they were returned to the laboratory within the day and processed on a dedicated bench. During each sampling event, temperature and oxygen concentration were recorded at each sampling site using a multiparameter probe (Hanna HI98129, Hanna Instrument).

Filtration was carried out using an electric vacuum pump and a 1L filtration unit (NalgeneTM) (Lawson Handley et al., 2019) on Sartorius® Nitrocellulose filters (47 mm in diameter for $0.45 \,\mu$ m pore size). Filtration continued until the filter clogged and the volume was then noted (Appendices A and B). The filter was removed, folded in quarters with sterilized tweezers, and stored in 1mL of 96% molecular grade ethanol at 4°C, under dark condition, until DNA extraction.

To avoid any station cross-contamination, all the equipment (tweezers and filtration units) was disinfected with 20% bleach and thoroughly rinsed with tap water. Then, 1L of distilled water was filtered between samples from each station to serve as a negative control.

2.2 | Seasonal and spatial eDNA sampling strategies

Seasonal variation of *A.pallipes* eDNA signal was assessed at three sampling periods presumed to be the major seasonal activity of white-clawed crayfish (Troth et al., 2021), in June, August, and October 2021 for the nine sampling sites selected: Milletiere (no 1), Ribault (no 2), Grand Vau (no 3), Peruzin (no 4), Duire (no 5), Courtineau (no 6), Quintaine (no 7), Rorthe (no 8), and Brule-Choux (no 9) (Figure 1, Appendix A). Milletiere (1) and Courtineau (6) are geographically closed but represent two independent rivers. Sampling protocols were the same as described above for each sampling period and each sampling site, to ensure homogeneous and comparable results.



FIGURE 1 Cartography of the study area (Indre et Loire French department), highlighting the nine sampling sites surveyed in the "Seasonal variation" experiment (black numbered dots) and the four "Spatial variation" stations, Le Long, Rorthe, Quintaine, Brule-Choux and Duire (red numbered squared). WILEY

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Spatial variation of A. pallipes eDNA signal was investigated through a downstream eDNA dispersal experiment, called "distance" hereafter. Previous studies have already shown the effect of river characteristics (velocity and discharge) and source biomass on downstream eDNA detection, often estimated between hundreds of meters to 2-3km in streams (Van Driessche et al., 2022; Wood et al., 2021). Distance experiments were prepared according to those estimated distance detection on 5 brooks where a total of 13 stations were sampled in August 2022: 3 in the Rorthe area (on A. pallipes downstream limit of the population, 1200 m downstream, and 2200 m downstream), 3 in Brule-Choux area (on downstream limit of the population, 350m downstream and 700 m downstream), 3 in Le Long area (on the downstream limit of the population, 1000m downstream and 2300m downstream), 2 in Quintaine area (on the downstream limit of the population and 300m downstream), and 2 in Duire area (on the downstream limit of the population and 700m downstream) (Appendix B). The downstream sampling sites were selected following their accessibility. The downstream limit of the crayfish populations was verified by intensive field sessions, for each station, in the weeks preceding the filtration campaign, with night observations (by light), and setting sardine-baited traps. The downstream limit of crayfish population, therefore, represents the most downstream point where an A. pallipes individual was observed and/or captured.

Unfortunately, the results of eDNA detection from the Brule-Choux "Spatial variation" station were found to be negative in all the samples collected in August 2022 by both qPCR and ddPCR analyses. Crayfish was known to be present at this station in 2021 (see experiment "seasonal variation," station no 9) (Figure 1) but no crayfish was observed in 2022 during field operation confirming its disappearance in this historic area.

2.3 | Primers and probe validation

Several primers and probe sets specific to the white-clawed crayfish have been developed and optimized within the literature (Table 1), targeting amplicon length ranging from 96 bp to more than 250 bp, and targeting a region of COI (Atkinson et al., 2019; Chucholl et al., 2021; Troth et al., 2020) or 16S genes (Manfrin et al., 2022). To choose the most suitable PCR set (primers and probe) for specimens found in France (our study area), we carried out an in-silico alignment using Geneious Pro R10 software (https://www.genei ous.com; Kearse et al., 2012), consisting in aligning these experimental primers and probe sets given in the literature (Table 1) with the genetic data of A. pallipes from French territory (from GenBank) (Appendix C). The experimental set giving the most satisfactory results (amplicon size < 120 bp and no mismatch) was the one developed by Atkinson et al. (2019). The others gave greater degrees of nucleotide incompatibility of the probe (Chucholl et al., 2021), or one of the two primers (Troth et al., 2020), some with excess mismatches (Manfrin et al., 2022).

The A. *pallipes* COI primers and probe (Forward: 5'-GGGTTAGT GGAGAGAGGGGT-3', Reverse: 5'-AATCCCCAGATCCACAGACG-3'

and 6-FAM probe 5'-TCAGCTATTGCCCACGCA-3') specificity was assessed by visual alignment using COI sequences of potentially co-occurring alien crayfish species (*Pontastacus leptodactylus*, *Procambarus clarkii*, *Pacifastacus leniusculus*) which could cross amplify (Appendix D). After in-silico validation, in-vitro specificity tests were performed using DNA extracted from the following species: *P. clarkii*, *P. leniusculus*, *Cherax quadricarinatus*, and *C. destructor*.

In-silico tests showed high specificity of primers and probe for *A.pallipes*, with no amplification of co-occurring or genetically closely related species. For example, when aligned with sequences *P.leptodactylus* (genetically closed species of *A.pallipes*; GenBank access number: KU571462 and KU571463), five mismatches were found in reverse primer and probe, and forward primer was not able to bind to the sequence (Appendix D). In-vitro tests, on DNA samples extracted from tissues showed no amplification either on tested species.

2.4 | DNA extraction from filters

DNA extractions were performed in a PCR-free dedicated room, separated from that used for the qPCR mix preparation. Before processing samples, all equipment and benches were bleach-disinfected. DNA extractions were performed on ¹/₄ section of the filter, using Qiagen DNeasy® Blood & Tissue commercial kit and following manufacturers guidelines with slight modifications described in Baudry et al. (2021), with a final elution volume of $50\,\mu$ L in AE Buffer. Extraction yields were checked by measuring DNA concentration using NanoDrop® 1000 Spectrophotometer (Thermo Scientific) and DNA samples were then stored at -20°C until further analysis.

2.5 | qPCR and ddPCR treatments

Each of the three biological replicates (i.e., water sample) was analyzed in duplicate by qPCR and ddPCR, giving a final number of six replicates of qPCR and 6 replicates of ddPCR per sampling station. Sample preparations were performed in a sterile room, decontaminated every night using UV-light treatment.

qPCR reactions were carried out in a 25 μ L final volume containing: 12.5 μ L of TaqMan® Environmental Master Mix 2.0 (Life Technologies, Applied Biosystems), 2 μ L of each primer (final concentration 1000 nM each), 1 μ L of probe (final concentration of 250 nM), 2.5 μ L of DNA-free water, and 5 μ L of DNA template. Amplifications were performed on a Roche LightCycler® 480 II quantitative thermocycler (Roche), using the following conditions: activation at 95°C for 10min followed by 55 cycles of 95°C for 15 s and 58°C for 1 min.

ddPCR reactions were also carried out in 25μ L final volume containing: 10μ L of ddPCR Supermix (no dUTP) 2×, 1.8μ L of each primer (final concentration 900 nM), 1μ L of probe (final concentration of 250 nM), 5.4μ L of DNA-free water and 5μ L of template. Droplets generation was performed by adding 70 μ L of "Droplet

 TABLE 1
 List of primers and probes specific to white-clawed crayfish Austropotamobius pallipes available in the literature.

References	Primer sequences	Probe sequences	Targeted gene	Amplicon length (bp)
Atkinson et al. (2019)	5'-GGGTTAGTGGAGAGAGGGGT-3' 5'-AATCCCCAGATCCACAGACG-3'	5'-6-FAM-TCAGCTATTGCCCACGCA-3'	COI	96
Troth et al. (2020)	5'GCTGGGATAGTAGGGACTTCTTT-3' 5'-CATGGGCGGTAACCACTAC-3'	5'-6-FAM-CTGCCCGGCTGCCCTAATTC-3'	COI	109
Chucholl et al. (2021)	5'- GAGGGTTAGTGGAGAGAGGG-3' 5'-AAATCCCCAGATCCACAGACG-3'	5'-CATCACT/ZEN/TTGCCCACGCAGG	COI	99
Manfrin et al. (2022)	5-CCCAGGAAATTTAAGCCTTTTCA 5'-TATTATGACCGTGCTAAGGTAGCA	5'- CAACCATTCATACCAGCCTTC	16S	136

Generation Oil for Probes" to each reaction and placed in the BioRad QX200 Droplet Generator. The emulsion was then transferred to a PCR plate sealed with a specific "Pierceable Foil Heat Seal" for 5s at 180°C in the BioRad PX1 PCR Plate Sealer heating block. Final PCR amplification was carried out using the BioRad C1000 Touch Thermal Cycler thermocycler using the following conditions: 10min at 95°C, 40 cycles of 30s at 94°C and 1 min at 60°C then 10min at 98°C. Results were finally read using the QuantaSoft software on the BioRad QX 200 Droplet Reader.

Each PCR plate (for both qPCR and ddPCR treatments) contained four negative controls (i.e. no-template DNA), to assess for potential contamination during the amplification, and four diluted positive standard samples (in duplicate for each concentration). These standards were generated using DNA extracted from *A. pallipes* tissue (40 ng/µL, quantified using a NanoDrop® 1000 Spectrophotometer) following the Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) Guidelines (Bustin et al., 2009), and concentrations ranged from 4×10^{-1} to 4×10^{-5} ng/µL. Positive signals were considered when a Ct value <42 (i.e., considered "false positive" if above), by qPCR and one positive droplet (setting the FAM amplitude level=2000), by ddPCR. A site was noted as "harbouring *A. pallipes*" if at least one replicate of the six technical replicates (per station and per PCR method) was positive (Atkinson et al., 2019; Bedwell & Goldberg, 2020).

2.6 | Limit of detection and limit of quantification

Limit of detection (LOD) and Limit of quantification (LOQ) were determined following MIQE guidelines requirement (Bustin et al., 2009), to compare the sensitivity of qPCR and ddPCR methods. LOD is the minimum concentration at which *A. pallipes* eDNA could be detected and LOQ is the minimum concentration at which its quantification was possible. To evaluate LOD and LOQ, DNA extracted from *A. pallipes* tissue was used to create 6 dilution series with concentrations ranging from $4 \text{ ng/}\mu\text{L}$ to $4 \times 10^{-6} \text{ ng/}\mu\text{L}$, and each of the 6 dilution levels was analyzed in 10 replicates by qPCR and ddPCR.

For qPCR method, LOD and LOQ were determined following Klymus et al. (2019) modeling, with slight modifications, as our concentrations were measured in ng/ μ L. Model was performed, for each PCR method, with "Best" parameter in the LOD.FCT and LOQ.FCT

functions, and 0.7 for the *LOQ.threshold* function, with a confidence interval <0.05.

For the ddPCR method, LOD was considered as the minimum concentration at which one replicate was positive (out of the 10 tested) and LOQ as the concentration at which nine replicates were positive (out of the 10 tested) (Mauvisseau et al., 2019).

2.7 | Inhibition tests

eDNA samples are known to be subject to potential inhibition due to the presence of natural compounds like Humic substances or Polyphenols in river water (Schrader et al., 2012; Takasaki et al., 2021).

To determine the level of inhibition in our eDNA samples, we followed the protocol used in Baudry et al. (2023), with some modifications. Briefly, we introduced 1µL of controlled DNA (4ng/µL) belonging to *C.quadricarinatus*, a crayfish species not occurring in France, in 4µL of environmental samples referred as "test" sample and 4µL of DNA-free water referred as "clean" control samples (with no inhibition). qPCR and ddPCR targeting *C.quadricarinatus* were performed in "test" and "clean" samples using dedicated primers et probe (Baudry et al., 2021) following the conditions described above.

This protocol was applied to each filter (biological replicate) used in this study and the inhibition level was determined for each station, by subtracting the average number of "test" cycles from the average number of "control" cycles. Inhibition was characterized if a Δ Ct>1 was observed (concerning qPCR treatment) and a Δ copy >10 (concerning ddPCR treatment).

2.8 | Analyses

Cartography was performed on QGIS 2.18 (Las Palmas) software (QGIS Team Development, 2016), with resources provided from the database ©IGN. All statistical analyses were performed using RStudio V1.1.463 (R Development Core Team, 2019, RGui v4.0.2). The normal distribution of the data and its homogeneity were verified with Shapiro-Wild and Bartlett tests.

Effects of covariates (temperature, oxygen, distance, and total volume filtered) on the probability of *A. pallipes* eDNA occurrence in

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water samples were modeled following MacKenzie et al. (2002) and Royle and Dorazio (2009) approaches. "eDNAoccupancy" R package was used, following procedures given in Dorazio and Erickson (2018) ("occModel" fitting determination and 10,000 iterations MCMC chains).

False negative errors were calculated using Griffin et al. (2019) modeling (https://seak.shinyapps.io/eDNA/), for qPCR and ddPCR, on samples collected for spatial variation (see above Section 2.2). The probability of species presence (ψ) was set at 1 for sites where *A. pallipes* was known to occur and fixed by the random parameters for sites sampled downstream (where the *A. pallipes* was unknown). Probabilities of eDNA occurrence in a PCR replicate (p) were investigated for qPCR and ddPCR, then both approaches were compared using Student's t-test.

The effects of seasonality and PCR method (qPCR/ddPCR) on the detection efficiency/probability were analyzed with a two-way ANOVA, with a significant probability of less than 0.05.

3 | RESULTS

3.1 | Sensitivity of the methods and inhibition test

During all PCR experiments (qPCR and ddPCR), no false positive (amplification of no-DNA samples) or false negative (no amplification of A. *pallipes* DNA standards) was reported.

During qPCR assays, LOD was found at a concentration of 3.65×10^{-5} ng/µL, with a mean Ct value of 39.94 ± 0.03 , and LOQ was found at a concentration of 9.78×10^{-4} ng/µL, with a mean Ct value of 36.82 ± 0.4 .

ddPCR seemed to perform correctly with 18,392±2139.8 droplets generated for each reaction. LOD and LOQ were respectively found at a concentration of 4×10^{-5} ng/µL (1.2 copy/5µL of eDNA) and 4×10^{-4} ng/µL (2.49±0.49 copy/5µL of eDNA), highlighting no difference between the two PCR methods.

No inhibition was found in our water samples as the maximum inhibition level (Δ Ct and Δ copy between "test" and "control" samples) observed was, respectively Δ Ct=0.53±0.08, for the "Duire" station and Δ copy=2.48±0.12. We can, therefore, affirm that the detection probability of A.*pallipes* eDNA was not affected by the inhibition phenomenon in our study, enabling the interpretation of our results regarding spatial and seasonal variation.

3.2 Environmental covariates influences

Occupancy modeling showed a significative effect of each environmental covariate tested (oxygen concentration, water temperature, and total volume filtered) on the probability of *A. pallipes* eDNA occurrence for both PCR methods (qPCR and ddPCR). The results were similar for each method, highlighting a significative positive influence of oxygen concentration (*p*-value < 0.01), a significative negative influence of temperature (*p*-value < 0.05), and a significative positive influence of total volume filtered (*p*-value < 0.05) on the probability of *A. pallipes* eDNA occurrence. eDNA occurrence probability reached 75% in sites where oxygen concentration exceed 8 mg/L^1 . For temperature and total volume filtered covariates, the eDNA detection probabilities remained very high, exceeding 80% when the measurements are below 20°C and 1000mL of total volume filtered (Figure 2).

3.3 | Spatial variation and false negative errors

Occupancy modeling results showed a significative negative influence of the distance from the *A. pallipes* population on the eDNA detection efficiency, for both qPCR and ddPCR methods (*p*value <0.05; Figure 3a,b). qPCR detection probability decreased <60% when water was sampled at a distance from *A. pallipes* population >1 km (Figure 3a). For the ddPCR method, eDNA detection probability decreased to 50% when water was sampled at a distance from *A. pallipes* population >1 km (Figure 3b).

All sites by sites results showed the same trend (Figure 4): for example, at Le Long site (1), *A. pallipes* detection probabilities were 83.3% using both qPCR and ddPCR methods, on samples collected at the site harboring the crayfish population, with Ct values and copy number of 40.4 ± 0.7 (per 5μ L of eDNA) and 2.34 ± 1.4 (per 5μ L of eDNA), respectively. Crayfish detection probability decreased to 33.3%, using both PCR methods, on water samples filtered at 2300m from the population, with lower Ct values and copies number (41.2 ± 0.05 per 5μ L of eDNA and 0.45 ± 0.7 per 5μ L of eDNA, respectively). Interestingly, *A. pallipes* detection probabilities were similar (only at Le Long site, using ddPCR method) or higher when water samples were taken 1000m downstream of the crayfish population for 1-Le Long and 2-Rorthe (Figure 4).

Concerning 3-Quintaine and 4-Duire sites, crayfish detection probabilities and sensitivity decreased for both approaches (qPCR, ddPCR) at lower distances than observed previously for 1-Le Long and 2-Rorthe sites: for the Quintaine site, after only 300m, *A.pallipes* detection probability was 13.3% using qPCR and the crayfish was undetectable using ddPCR method (Figure 4). For the Duire area, crayfish detection probabilities decreased <50% when water samples were taken at 700m downstream of the crayfish population (Figure 4).

Modeling highlighted high probabilities of eDNA occurrence (>0.8) at the downstream site (ψ), for each station, even those >2000m (Table 2). But a decrease in this probability of eDNA occurrence (ψ) was observed as we sampled downstream the crayfish population (Table 2). The "Probability of eDNA occurrence in a PCR replicate" (p) modeling results highlighted a significatively higher sensitivity of the qPCR method (t=21.102, p-value <0.001) and a decreased probability of eDNA detection as both qPCR and ddPCR

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FIGURE 2 Influence of (a) oxygen concentration, (b) temperature, and (c) total volume filtered on the probability of Austropotamobius pallipes eDNA occurrence after a qPCR analysis, (d) oxygen concentration, (e) temperature, and (f) total volume filtered on the probability of Austropotamobius pallipes eDNA occurrence after a ddPCR analysis; following site occupancy modeling.

methods were applied on water samples collected downstream (Table 2).

3.4 | Effect of seasonality

The two-way ANOVA results performed on the 162 samples (nine sites, three biological replicates, two technical replicates) showed a positive effect of the season on the detection efficiency of *A. pallipes* (p < 0.01; Table 3). The PCR method (qPCR or ddPCR) had no significant effect on the crayfish detection (p = 0.895) as well as the cumulative effect of the "season AND the PCR method" (p=0.798).

The probability of crayfish detection, as well as the signal intensity (Ct value for qPCR or copy number for ddPCR) were significantly higher in August than in June and October for the two PCR methods (Figure 5). In August, the detection probabilities of *A. pallipes* reached 90.7 \pm 18.8% for qPCR and 83.3 \pm 28.9% for ddPCR, with Ct values reaching 36.8 \pm 2.6 and copy number 40.4 \pm 72.2 copies per 5µL (Figure 5). In June, the detection probabilities of *A. pallipes* reached 55.6 \pm 39.1 using qPCR and 63 \pm 38.9% using ddPCR, with Ct values of 38.2 \pm 1.8 and copy number reaching 10.9 \pm 9.9 copies per 5µL (Figure 5). Finally, in October the detection probabilities of *A. pallipes* decreased to 44.4 \pm 37.2% using qPCR and 40.7 \pm 38.3% using ddPCR, with Ct values of 38.9 \pm 1.6 and copy number reaching 2.1 \pm 5.4 copies per 5µL (Figure 5).

4 | DISCUSSION

In this study, we evaluated the operationality of eDNA-based approaches as a conservation tool to monitor the endangered crayfish A. pallipes populations in rivers. Prior evaluating spatial, seasonal, and potential effects of environmental covariates on eDNA detection, we applied a rigorous validation procedure to improve the interpretation of our results. We confirmed in silico and in vitro that the most adapted A. pallipes gPCR and ddPCR primers and probes available in the literature were the ones proposed by Atkinson et al. (2019), targeting a 96 bp long region of the COI gene. We evaluated the LOD and the LOQ of our gPCR (Klymus et al., 2019) and ddPCR (Mauvisseau et al., 2019) assays, which are key factors to determine the limits of eDNA approaches and improve the confidence of ecological interpretation. Another aspect we evaluated was the potential effect of environmental PCR inhibitors known to affect particularly qPCR approaches (Lance & Guan, 2019). As no inhibition effect was observed in our environmental samples, qPCR and ddPCR results were not influenced by inhibitors in our context. If we refer to the eDNA approach validation scale proposed by Thalinger et al. (2021), we reach a high level of validation for our eDNA speciesspecific assays. Thus, our validation procedure was performed for A. pallipes but could easily be reproduced in other crayfish taxa. Our results highlighted seasonal and spatial variation in eDNA detection efficiency. We determined an optimal sampling period for the eDNA



FIGURE 3 Effect of sampling distance from the Austropotamobius pallipes population on the probability of A. pallipes eDNA detection, following occupancy modeling.



FIGURE 4 Cartography of the "Spatial variation" results investigated at the four considered areas, Le Long, Rorthe, Quintaine, and Duire (red numbered squared).

	Distance	Probabi of eDN/ Occurre site (ψ)	lity A ence on	Probabi of eDNA Occurre PCR rep	lity A Ince in a Ilicate (<i>p</i>)
Sites	(m)	qPCR	ddPCR	qPCR	ddPCR
Le Long	0	1	1	0.874	0.767
	1000	0.899	0.932	0.86	0.701
	2300	0.827	0.888	0.808	0.669
Rorthe	0	1	1	0.818	0.78
	1200	0.907	0.935	0.675	0.546
	2200	0.827	0.888	0.64	0.481
Quintaine	0	1	1	0.797	0.503
	300	0.827	0.888	0.738	0.297
Duire	0	1	1	0.781	0.692
	700	0.8	0.888	0.73	0.57

TABLE 2Probability of eDNA occurrence calculated followingDorazio and Erickson (2018) and Griffin et al. (2019).

TABLE 3 Two-way Analysis of Variance (ANOVA) investigating the effects of seasonal effect and PCR method used on the detection efficiency of *Austropotamobius pallipes*, using ddPCR and qPCR eDNA methods.

Source of variation	Sum of square	df	F	p-value
Season	18,148	2	7.6863	<0.01 **
Method (qPCR vs. ddPCR)	21	1	0.0174	0.895
Season×method	535	2	0.2266	0.798
Residuals	56,667	48		

Note: ** symbols mark the significance of the results.

detection of A. pallipes as well as the distance to eDNA source assumption to give advice regarding field sampling strategies.

4.1 | Effects of environmental covariates

For both qPCR and ddPCR methods, the crayfish detection probability was significatively and positively influenced by the water oxygen concentration and the total volume filtered, and negatively influenced by the water temperature. Water temperature and its oxygen concentration are well known to be correlated (not obligatorily significantly, as in this study) and an increase in temperature is known to significatively accelerate the degradation of eDNA fragments (Saito & Doi, 2021). As an example, Moyer et al. (2014) showed a decrease in detection efficiency up to 1.7 times, when the temperature increases by 1°C.

The results showed a positive and significant correlation between the total filtered volume and the detection probability of *A. pallipes* eDNA. This is reassuring given that many recent studies seek to optimize protocols to filter ever more water, up to 100L (Valentini et al., 2016) or even from 1000 to 3000L (Schabacker et al., 2020). However, these studies use filtration methods different from those used here and if we compare similar protocols, other studies have also demonstrated an efficiency up to 5 times higher when 2 L of water were filtered instead of one, in a lotic environment (Bedwell & Goldberg, 2020).

Nevertheless, it would therefore seem that here, these results were mainly guided by the ecological preferences of our target species, A. pallipes. The white-clawed crayfish is a species that preferentially occurs in headwater areas (Souty-Grosset et al., 2006; Trouilhé et al., 2007). These headwater areas, in the temperate zone, are characterized by colder and well-oxygenated waters, unlike the downstream zones, where the temperature increase, and the oxygen concentration decrease, with the drop in altitude (Trouilhé et al., 2007). These areas at the head of the watersheds are often also mostly characterized by rather clear water, with a low organic matter content (Tamooh et al., 2012), which, therefore, logically allows larger volumes of water filtered before membrane clogging. It was therefore not surprising to obtain results demonstrating a high probability of detection of A. pallipes in these highly oxygenated and low-tempered areas, where larger volumes of water could be filtered.

4.2 | Spatial variation

From the four sampling areas used to investigate the influence of distance from source populations on the eDNA detection, the results showed a decrease in both the probability of detection, by calculation (Figure 4) and by modeling (Table 2), and the detection efficiency (for ddPCR and qPCR), when the sampling sites were downstream the A.pallipes population. The interpretation of these spatial results is guite challenging to discuss because the detection distance is clearly dependent on many biotic, abiotic, and experimental factors, such as eDNA source depending on taxa/ species and biomass, river hydrodynamics, decay rates depending on environmental factor like temperature and UV, volume filtered. Several studies have focused on the study of the eDNA dispersal distance, considering these different factors, in lotic environments, via experimental caged-species systems, field observation, and/or modeling (Baudry et al., 2023; Nukazawa et al., 2018; Van Driessche et al., 2022; Wacker et al., 2019). Certain trends seemed to converge, and to fit with our results (for the eDNA dispersal distance): the eDNA quantity released at the source, correlated to the biomass of target individuals and their activity, including molting-for crustaceans-and breeding, has a positive influence on its downstream dispersion, with reliable detection up to 2 to 3km, on different fish species (Baudry et al., 2023; Nukazawa et al., 2018; Van Driessche et al., 2022) and bivalves (Wacker et al., 2019).

Crayfish, in general, are well known to release very little eDNA quantities into the environment, because of their morphology, composed of an exoskeleton and a cuticle, releasing little mucus in the water (Dougherty et al., 2016). White-clawed crayfish populations are also often of low densities, fragmented in some places, or locally



FIGURE 5 Graphical representations of (a) detection probability of *Austropotamobius pallipes* by qPCR and ddPCR methods; and sensitivity of (b) the quantitative PCR method (Ct values) and (c) the digital droplet PCR (Log copies number/5 µL), through three seasonal periods (June, August, and October).

extinct (Collas et al., 2016; Füreder et al., 2010), reducing the eDNA source emission. Nevertheless, here, detection of crayfish remained possible up to more than 1 km and even 2 km in some cases, representing very encouraging results in the context of *A. pallipes* population monitoring, especially in this area, where the brooks sampled are small, narrow, and shallow, certainly harboring low population densities. By sampling favorable areas, within a linear distance of approximately 2 km, it becomes possible to detect the crayfish using eDNA method, and to delimit areas to sample by the traditional way (observation or trapping).

4.3 | Seasonal variation

Our experiments showed a significative influence of the season on the *A. pallipes* detection efficiency and probability, which is in accordance with other studies on the same species (Dunn et al., 2017; Troth et al., 2021). This is not surprising as crayfish activity is known to change according to the water temperature (and so, seasons), ranging from low activity/torpor in winter, juvenile hatching in May/ June, high activity level and molting in summer, and breeding before winter (October/November) (Troth et al., 2021). Higher activity levels and molting in August, seem to lead to a higher amount of eDNA in the water and better detection by PCR methods. However, contrarily to our results, Dunn et al. (2017) and Troth et al. (2021) highlighted a better white-clawed eDNA detection in June, during the juveniles emergence period. But these two cited experiments were run under controlled conditions, without considering variations in water levels observed in the wild. This can explain the difference with our study, where the water was sampled during the low water period, in August.

In addition to these periods of high organism activity, these water levels and/or flow rates have an important role in the eDNA detection efficiency, as demonstrated by Curtis et al. (2020), for the Asian corbicle (*Corbicula fluminea*). During the summer period, the water levels (and the flow rates) of the rivers decrease (linked

pling from year to year. This phenomenon has a considerable impact on water temperature, precipitation, and the hydrological regime (Graham et al., 2007), and seems to be discontinuous, with some inter-annual variations possible. With this climate change, suitable areas for *A. pallipes* tend to decrease (Préau et al., 2020) and periods of severe drought to become more and more frequent, with high water temperatures in summer, making eDNA detection yields questionable. It is therefore difficult to predict, to the nearest month, the optimal period for eDNA sampling in an *A. pallipes* population monitoring context, but we suggest sampling when water temperatures are below 18–20°C.

4.4 | qPCR versus ddPCR

We compared two PCR methods commonly used for eDNA species detection surveys (gPCR and ddPCR), with a known bias, with no differences being apparent in our study context. These results contrast with other studies, which showed a greater efficiency of ddPCR for eDNA detection (Doi et al., 2015; Wood et al., 2019). For example, Wang et al. (2022) systematically compared the two PCR methods (qPCR and ddPCR) for quantitating both bacteria and fungi, in test samples (mock communities) and environmental/ field samples. Under test conditions, ddPCR was found to be significantly closer to expected values, with less variation between replicates (p-value < 0.05). These same observations were made in the field, highlighting a better precision, repeatability, sensitivity, and stability in bacterial and fungal quantitation than gPCR. This study, even if it appears to be very robust, relates to the detection of particular species (bacteria and fungi), very different from animal species, such as crayfish (Wang et al., 2022). The detection of crustaceans is known to be relatively challenging: they shed less eDNA than other organisms in the water (Allan et al., 2021), probably due to their exoskeleton. This phenomenon was accentuated in case of low density of population, as may be the case for A. pallipes, resulting in weak eDNA signals using PCR methods. Certain environmental parameters must be considered, such as the presence of PCR inhibitors due to the high amount of suspended matter in the water.

However, in our study, we demonstrated an absence of inhibition in our samples, resulting in similar yields from the two PCR methods. This absence of inhibition can be due to the use of Taqman Environmental Mastermix 2.0 during our PCR experiments: Strand et al. (2011) showed the great efficiency of this mix, able to remove close to 100% of the observed inhibition in eDNA extracts, including from water with high content of humic substances. Considering these similar detection efficiencies, we recommend the qPCR approach in the context of *A. pallipes* monitoring populations in France, under our experimental conditions. In our study context, qPCR, in addition to allowing a very satisfactory detection of crayfish, turns out to be less expensive and less time-consuming than ddPCR. Regarding the price, it is dependent on the cost of the reagents, which can be negotiated differently depending on the geographical areas and the laboratories considered. For our study, the cost of one eDNA sample analysis (for *A. pallipes* detection) is $17 \notin 60$ for qPCR against $30 \notin 50$ for ddPCR (from the extraction of eDNA, from filters, to the results visualization, without considering all the standard costs, such as equipment depreciation and human time).

qPCR also has an advantage over ddPCR regarding the experimentation time, something on which all the studies carried out agrees (Doi et al., 2015; Wang et al., 2018, 2022). No difference occurs in the eDNA extraction and preparation phases, both methods using similar protocols and similar concentrations of eDNA, but in the PCR reactions preparation and results visualization. For the two PCR methods, the mixes are relatively similar, not necessarily in terms of reagents and their concentrations but in terms of preparation time. However, while qPCR only requires plate filling, with mixes and templates (eDNA, DNA-free samples, standards, and negative controls, etc...), it is necessary to generate droplets for ddPCR, using the BioRad QX200 Droplet Generator, a step that can take more than 30 min per plate. The amplification protocols, once the generation of droplets has been completed for ddPCR, are the same for both methods (gPCR and ddPCR) and therefore last the same time. But a new difference appears when visualizing the results: qPCR (using a Roche LightCycler® 480 II quantitative thermocycler) allows live visualization of the results, while for ddPCR it is necessary to read the results, when the PCR amplification run was completed, using the BioRad OX 200 Droplet Reader, a new step that can last more than 1h per plate.

5 | CONCLUSION

We adapted and optimized an eDNA-based approach allowing the detection of the endangered white-clawed crayfish A. pallipes in French watercourses. Improved laboratory procedures allowed a confident validation of the method and the detection of the species in the natural environment. It allowed detection of the crayfish species up to 2km downstream of the population, even in context with low densities, considering this endangered species. Thus, eDNA could be a useful tool for freshwater managers and stakeholders to refine the presence of crayfish on large number of sites, in order to orient precisely conventional field surveys (e.g., population genetics, counting). This method proved its reliability for A. pallipes population monitoring, unfortunately highlighting the disappearance of one population between 2021 and 2022 (Brule-Choux). Even if the summer period seems to be the most conducive to the A.pallipes detection using the eDNA method, it would seem judicious to adapt the sampling from year to year, given that these seasons are bound to change, in a growing context of climate change. Finally, we recommend to stakeholders a detection approach by qPCR, under our

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experimental conditions, offering very satisfactory results, at costs almost two times lower than those of ddPCR (proven to be of equal performance).

AUTHOR CONTRIBUTIONS

Thomas Baudry: methodology, software, formal analysis, data curation, writing-original draft, writing-review and editing, visualization, and manuscript validation; Maud Laffitte: lab experiments, data curation, writing-review and editing, and manuscript validation; Charlotte Noizat: lab experiments, data curation, and manuscript validation; Carine Delaunay: lab experiments and manuscript validation; Grégoire Ricou: field investigation, project administration, funding acquisition, and manuscript validation; Valentin Vasselon: writing-review and editing, visualization, and manuscript validation; Frédéric Grandjean: conceptualization, supervision, project administration, funding acquisition, and writing-review and editing.

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CONFLICT OF INTEREST STATEMENT

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.

DATA AVAILABILITY STATEMENT

All data generated or analyzed during this study are included in this published article (and its supplementary information files) and additional information and data are available from the corresponding author upon reasonable request.

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ported by month (June, August, and October) with the qPCR and ddPCR position proportion of replicates and the sensitivity of the positive signal (Mean Ct for qPCR and Mean copy number for eDNA collection locations including field data (GPS coordinates and total volume filtered) for each of the nine sites prospected for the "Seasonal variation" experiment, in 2021. Results are reddPCR). A (-) symbol represents no amplification of Austropotamobius pallipes eDNA.

		June 2021			August 2021			October 2(021	
Sampling site	Coordinates (X, Y)	Total volume filtered (mL)	qPCR positive proportion (mean Ct)	ddPCR positive proportion (mean copy number)	Total volume filtered (mL)	qPCR positive proportion (mean Ct)	ddPCR positive proportion (mean copy number)	Total volume filtered (mL)	qPCR positive proportion (mean Ct)	ddPCR positive proportion (mean copy number)
Milletiere	515,783, 6,672,481	1150	1 (36.45)	1 (22.23)	1300	1 (35.93)	1 (28.72)	1100	0.67 (37.31)	0.83 (9.59)
Ribault	526,836, 6,654,425	1300	0.83 (37.67)	1 (5.44)	1400	1 (34.2)	1 (200.86)	1100	(-) 0	0.33 (0.68)
Grand Vau	533,890, 6,644,870	1200	1 (36.91)	1 (14.4)	1600	1 (37.19)	1 (29.37)	1300	1 (38.65)	0.83 (4.72)
Peruzin	555,412, 6,678,601	006	0.17 (41.1)	0.5 (4.18)	1600	1 (39.65)	0.83 (10.21)	1100	0.33 (38.94)	(-) 0
Duire	509,699, 6,725,506	1100	0.33 (40.53)	0.5 (1.91)	1500	1 (38.53)	1 (18.14)	1100	0.33 (40.31)	0.67 (1.71)
Courtineau	520,406, 6,673,441	006	0.17 (40.24)	0.17 (1.59)	1200	0.5 (39.97)	0.33 (3.32)	006	(-) 0	(-) 0
Quintaine	543,987, 6,718,225	600	0.17 (41.09)	0.5 (1.91)	700	1 (37.43)	1 (14.69)	700	0.33 (39.89)	0.17 (3.03)
Rorthe	525,665, 6,734,053	1050	0.33 (41.02)	(-) 0	1400	0.67 (40.87)	0.33 (1.495)	1100	0.33 (41.03)	(-) 0
Brule-Choux	493,269, 6,726,406	850	1 (38.76)	1 (7.86)	1000	1 (34.83)	1 (59.55)	750	1 (38.64)	0.83 (3.26)

APPENDIX B

eDNA sampling data (with distance from Austropotamobius pallipes population) including field data (GPS coordinates, total volume filtered, temperature, and oxygen concentration) for each site prospected for the "Spatial variation" experiment, in 2022. Results of qPCR and ddPCR are reported with the proportion of positive replicates and the sensitivity of the positive signal (Mean Ct for qPCR and Mean copy number for ddPCR, ±standard deviation). A (-) symbol represents no amplification of Austropotamobius pallipes eDNA.

	Dictance from					qPCR		ddPCR	
Sampling area	A. pallipes population (m)	Coordinates (X, Y)	Total volume filtered (mL)	Temperature (°C)	Oxygen (mg/L)	Proportion of positive replicates	Mean Ct±SD	Proportion of positive replicates	Mean copy number±SD
Le Long	0	521,646, 6,720,824	600	16.9	8.3	0.83	40.45 ± 0.75	0.83	2.34 ± 1.45
	1000	521,479, 6,721,872	006	17.8	8.4	1	40.18 ± 0.99	0.83	1.78 ± 1.42
	2300	520,537, 6,722,607	1050	17	7.5	0.33	41.16 ± 0.06	0.33	0.45 ± 0.69
Rorthe	0	525,665, 6,734,053	1800	16.3	8.9	0.83	39.72 ± 0.95	0.83	1.43 ± 0.88
	1200	524,029, 6,734,149	1800	16.7	6.5	1	37.78 ± 0.36	1	5.48 ± 2.77
	2200	523,026, 6,764,343	1800	16.9	80	0.5	41.17 ± 0.68	0.33	0.66 ± 1.14
Quintaine	0	543,987, 6,718,225	2100	20	6	0.5	40.26 ± 0.46	0.67	1.98 ± 1.76
	300	543,934, 6,717,850	2100	21.2	7.9	0.17	40.98	0	ı
Duire	0	509,699, 6,725,506	1800	16.8	11.2	0.5	40.99 ± 0.91	0.5	0.44 ± 0.69
	700	510,199, 6,725,786	1800	17.4	10.7	0.33	41.3 ± 0.57	0.33	0.41 ± 0.66

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APPENDIX C

Example of alignment of primers and probes developed by Atkinson et al. (2019), Troth et al. (2020), Chucholl et al. (2021), and Manfrin et al. (2022) with a DNA sequence of *Austropotamobius pallipes* (here access number AY667115), performed on Geneious Pro R10. It can be seen that only the Forward primers PallCOIF (developed by Chucholl et al., 2021) and COI-F (developed by Atkinson et al., 2019), the Reverse primers WC2302R (developed by Troth et al., 2020, 2021), PallCOIR (developed by Chucholl et al., 2021) and COI-F (developed by Atkinson et al., 2019) and the AppMGB probe (developed by Atkinson et al., 2019) were able to hybridize correctly.



APPENDIX D

Example of sequences alignment of co-occurring species in the wild with *Austropotamobius pallipes* with the PCR set (primers and probe) developed by Atkinson et al. (2019), performed on Geneious Pro R10. The species sequences were found on GenBank (in this example *Pontastacus leptodactylus*: KC789387, KC789391, KU571462, KU571463.1, KU603529; *Procambarus clarkii*: KP976367, KP976368, KT959364.1, MF170535, MF170536, and *Pacifastacus leniusculus*: ON059119, ON059120). Here, only the Reverse primer and the probe of the set hybridize with 2 sequences (of *P. leptodactylus*), highlighting 2 mismatches (in yellow) for the probe and 3 mismatches for the reverse primer.

