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## RESEARCH ARTICLE

WILEY

# Environmental DNA survey to detect an endemic cryptic fish, *Anablepsoides cryptocallus*, in tropical freshwater streams

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## Abstract

1. Martinique island is a biodiversity hotspot in the Lesser Antilles that harbours many endemic species. One of these, *Anablepsoides cryptocallus*, also known as 'Poisson gale', is the only endemic freshwater fish of Martinique and a species with a poorly understood distribution range.
2. In this study, an environmental DNA (eDNA) detection protocol was developed, validated, and optimized, targeting a short fragment (125 bp) of the *A. cryptocallus* COI mitochondrial gene, to investigate the presence of this species in Martinique. Fifty-seven sites spread over 43 permanent rivers and two wetlands were sampled using both eDNA and conventional fishing (dip net capture).
3. Presence was confirmed in 27 sites using eDNA detection, and in nine sites by dip net fishing. eDNA-based detection of *A. cryptocallus* was effective and less time-consuming than conventional fishing, making it a relevant tool for future studies throughout the island. Even though *A. cryptocallus* was found to be present in a significant number of sites, many sites previously known for this species were found to be negative, highlighting the need for continuous monitoring.
4. There is now an urgent need to propose protection status for this endemic species to preserve its preferential habitats, as these are being increasingly threatened by human activities that are leading to habitat loss and fragmentation.

## KEYWORDS

endemic species, environmental DNA, freshwater, Martinique, real-time PCR, tropical environment

## 1 | INTRODUCTION

Human activities through agriculture (e.g. crop rotation and use of pesticides), the development of infrastructure (e.g. roads, canals, dams) and damage to aquatic environments (e.g. by dredging, draining

and barriers) are leading to a global reduction in the health of all ecosystems, resulting in a considerable erosion of their biodiversity (Wilcove et al., 1998; Hautier et al., 2015; Ferreira et al., 2017). Current species extinction rates are estimated to be about 100 times higher than the average extinction rate during previous major crises in

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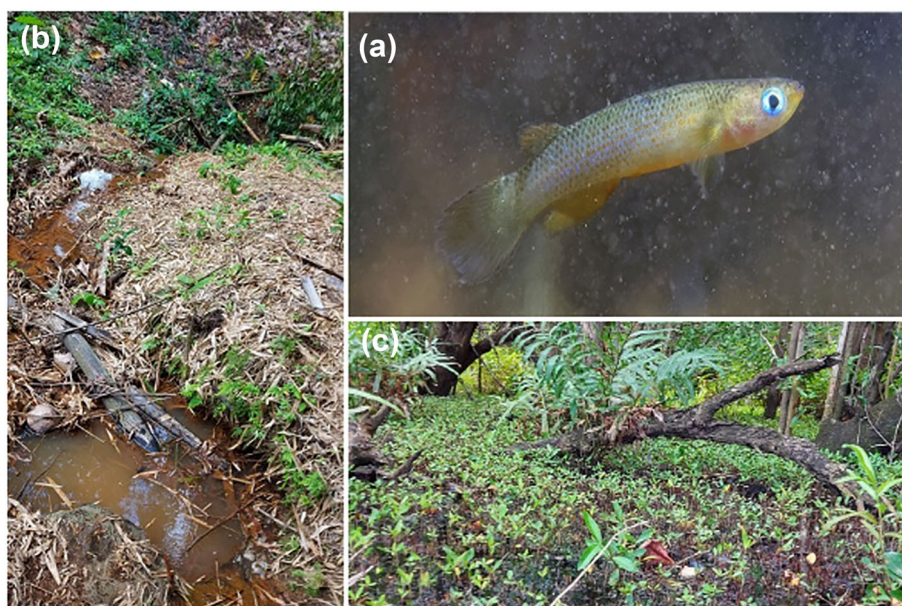
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geological time, and the present biodiversity crisis is now recognized as the sixth species extinction crisis (Ceballos et al., 2015; Unnerstall, 2022). The loss of biodiversity has impacts on ecosystem structures and modifies their functional equilibrium, in turn affecting their capacity for resilience to disturbances. Thus, these ecosystems become more fragile and less resistant to biological invasions, a major factor in current biodiversity loss linked to increases in global exchange. These impacts are even more rapid and more significant in island environments. Islands often contain smaller populations that are very sensitive to environmental disturbances, making their ecosystems very fragile (Vitousek, Loope & Westbrooks, 1996; Myers et al., 2000). There is therefore an urgent need to set up environmental protection programmes to preserve their biodiversity and their ecological functions (Flitcroft et al., 2019). However, to establish such programmes, it is necessary to have a good knowledge of these environments, as well as the various species that they harbour. This requires inventories, with accurate species identification and assessment (endemic, rare, endangered or invasive) both to inform policy-makers for the protection of these environments, and to assess the health of the ecosystems and the species that inhabit them.

Martinique is a rugged island of 1128 km<sup>2</sup>, located in the Lesser Antilles archipelago in the Atlantic Ocean. Despite its small size, it has a wide variety of landscapes and terrestrial ecosystems with more than 70 permanent rivers – as well as many non-permanent ones, tropical forests wetlands and mangroves (DEAL Martinique, ECOVIA & CREOCEAN, 2018). Its diverse ecosystems host a wide variety of flora and fauna, with an overall high endemism rate for the Antilles (DEAL Martinique, ECOVIA & CREOCEAN, 2018). Among the Antillean endemics, *Anablepsoides cryptocallus*, better known by its vernacular name 'Poisson gale', is the only endemic freshwater fish in Martinique (Lim et al., 2002) (Figure 1a). It belongs to the family Rivulidae and is mainly found in aquatic systems of varying sizes such as ditches, pools and backwaters (Figure 1b and c), characterized by a weak current (or even an absence of current) and a dense riparian

cover (Lim et al., 2002). However, the ecological requirements of this fish remain poorly understood. In 2019, a study commissioned by the Direction de l'Environnement, de l'Aménagement et du Logement (DEAL) of Martinique and the Office de l'Eau (ODE) of Martinique was conducted to establish a potential distribution map of the species based on bibliographic research, modelling of its preferred habitat and field surveys using dip nets. Information on the preferred habitats (supplementary information; Appendix A – Biotope, 2020) is highlighted as 'hotspots', i.e. areas where the environmental characteristics lead to a higher detection probability of the species considering the following criteria: cloud cover, height of vegetation, distance from woodland, altitude and slope. These areas were mainly located in the centre of the island and on the North Atlantic coast, where the species was historically recorded. Subsequent dip net fishing at previously known and predicted sites confirmed its presence in 10 sites but also suggested its disappearance from some of the historically known sites, particularly on the North Atlantic coast (Biotope, 2020). A further documentary search and collection of testimonies from local fishermen revealed the existence of 25 additional sites where *A. cryptocallus* potentially occurred. Nevertheless, its preferred habitat of ditches and wetlands makes it highly sensitive to impact from human activities such as ditch cleaning and the destruction of wetlands, both of which are common in Martinique.

In recent years, biological inventories have undergone a revolution, particularly in aquatic environments, through the development of monitoring techniques based on the detection of DNA released by organisms present in the water, called 'environmental DNA' (or eDNA). It is based on the detection of organisms from pieces of skin, eggs or mucus released into their environment at any stage of life, without the need to observe the target species physically (Ficetola et al., 2008; Thomsen & Willerslev, 2015). Monitoring using eDNA is very effective for the early detection of cryptic, rare, endemic and threatened fish species (Goldberg et al., 2016; Piggott, 2016; Brys et al., 2020) and has been applied widely in recent years, even in tropical areas (Cantera



**FIGURE 1** Photographs of the unique endemic freshwater fish species in Martinique, *Anablepsoides cryptocallus* (a), and examples of its preferred habitats: a ditch in Gros Morne station (b) and a backwater forest in François station (c).

et al., 2019; Baudry et al., 2021). Application of this technique could considerably improve knowledge on the distribution of *A. cryptocallus* in Martinique. Indeed, this species is difficult to detect and capture owing to its cryptic and elusive nature, small size (around 6 cm maximum), and ability to bury itself in the mud during the dry season (Lim et al., 2002). In addition, these fish are often located in remote upstream areas that are sometimes not easily accessible. Thus, the development of eDNA monitoring for this fish should allow its presence in oxbows or ditches to be confirmed by sampling from the main river bed, because DNA can be characterized a few hundred metres downstream from where a species is located (Deiner & Altermatt, 2014).

The objective of this study was to develop and assess the efficiency of eDNA detection to monitor the presence of *A. cryptocallus*, compare its reliability with a conventional method (dip net capture) and update the knowledge on its distribution in Martinique. In addition, the study aimed to generate the first distribution map of this endemic species in Martinique, thereby identifying which areas should be preserved, as well as to guide future monitoring in the context of upcoming genetic studies. To achieve this, species-specific primers were designed and tested, followed by eDNA monitoring at a range of field sites.

## 2 | MATERIAL AND METHODS

### 2.1 | *In-situ* sampling protocol

In total, 57 sites spread over 43 permanent rivers and two wetlands were surveyed. The choice of sites was based on historically known presence, but also on preliminary knowledge regarding the habitat preferences of *A. cryptocallus* (Biotope, 2020) (Supplementary information; Appendix A). Sites where *A. cryptocallus* was recorded in the Biotope (2020) study were also investigated, to serve as positive *in-situ* controls. All sites were sampled by conventional fishing, dredging the banks and aquatic macrophytes with a dip net. Each microhabitat (e.g. roots, mud, rocks) in the site was sampled for a maximum of 5 min each. If no catches were made in a total of 30 min at the site, it was considered not to harbour *A. cryptocallus*.

Water samples were collected according to the method described by Baudry et al. (2021). Water was collected from one river bank to the other in a sterile plastic bottle using gloves (Coward et al., 2018), and then filtered in the field, directly following sampling. The water was poured into a Nalgene filtration unit (Lawson Handley et al., 2019) containing nitrocellulose filters (Sartorius 47 mm diameter and 0.45 µm pore size). Filtration took place until the filter was saturated with the suspended solids present in the water. The volume filtered was then recorded (Supplementary information; Appendix B), and the filter folded into quarters and preserved in tubes filled with molecular grade absolute ethanol. Three biological replicates (three filters) were collected at each site. To avoid cross-contamination between sites, all equipment was decontaminated using a 50% bleach solution after each site and thoroughly rinsed with tap water. At each site, a control sample (1,000 ml of distilled water)

to test for potential contamination between the different surveyed locations was filtered in the same way as described above before filtering water from the site. All samples were stored in a cooler until they were returned to the laboratory and stored at -20 °C before analysis.

### 2.2 | DNA extraction from filter

All extractions were carried out in a dedicated room, where the benches were disinfected every night by UV treatment to avoid any risk of contamination. All surfaces, equipment and tools were sterilized using a 50% bleach solution.

Following the protocol in Baudry et al. (2021), a quarter of a filter was cut and dried for 15 min to evaporate the ethanol. A Qiagen DNeasy Blood & Tissue Kit DNA extraction kit (Düsseldorf, Germany) was used to isolate the total DNA from each filter, following the manufacturer's recommendations with some modifications, as in Baudry et al. (2021). Extraction yields were then measured using the NanoDrop 1000 spectrophotometer (Thermo Scientific, Wilmington, DE, USA; Supplementary Information, Appendix B) and the extracted samples were stored at -20 °C until analysis by real-time PCR (Roche LightCycler 480 II, Basel, Switzerland).

### 2.3 | qPCR assays

The primers and probe were designed using Geneious Pro R10 software (<https://www.geneious.com>; Kearse et al., 2012), based on the sequences present in GenBank (Supplementary Information; Appendix C) and following the method outlined in Brys et al. (2020). Using sequences isolated from a portion of the COI mitochondrial gene (Biotope, 2020), a set of species-specific primers and a probe targeting a 125 base-pair fragment of the COI gene was developed (forward 5'-AATAATTGGAGCCCCTGACA-3'; reverse 5'-TTCACCCTGTTCTGCTCTCT-3'; and 6-FAM MGB probe 5'-ACTTTTACCCCTCTTCT-3'). Using the Geneious 'Primer Design' function, their specificity was assessed by visual alignment using COI sequences of co-occurring fish and crustacean species from the West Indies and genetically close species. *In-silico* tests were done using the NCBI primer projection tool (<https://www.ncbi.nlm.nih.gov/tools/primer-blast/>).

Following *in-silico* validation, the set of primers/probe was tested at different concentrations (from 0.5 to 10 µM final concentration) and different annealing temperatures (from 55 to 61°C). Specificity was tested *in-vitro* with real-time PCR, under optimal conditions, using DNA extracted from fish species co-occurring in Martinique and/or genetically close: *Hypostomus plecostomus*, *Ancistrus* sp. and *Hypostomus robinii* (three species of the family Loricaridae, highly valued in aquaria, including *H. robinii*, occurring naturally in Martinique), *Poecilia reticulata* (a species of guppy, introduced in Martinique) and *Kryptolebias marmoratus* (a species of the family Rivulidae occurring naturally in Martinique).



## 2.4 | Mesocosms

Mesocosm experiments were carried out to assess the efficiency of the primers on eDNA extracted from known positive samples. Two conditions were tested: two specimens (one male and one female) in 2 L of water and two specimens (one male and one female) in 50 L of water. The specimens were left for 1 week in the aquaria as an accommodation period during which eDNA could diffuse into the water. The water temperature and pH were maintained at 27°C and 7.5 respectively, in line with the values found in the preferred environment of *A. cryptocallus*, with a natural 12/12 h photoperiod. After a week, eDNA samples were taken from the mesocosm, and water was filtered into two biological replicates (two filters) for each condition following the method described above. Two real-time PCR replicates (technical replicates) were performed on each biological replicate.

## 2.5 | qPCR analyses

Two real-time PCR replicates were performed on the eDNA extracted from the filters using a LightCycler® 480 II quantitative thermocycler (Roche, Basel, Switzerland), at the determined optimal conditions. These were carried out on the extracted DNA from each filter, and this corresponded to six amplifications per site (i.e. three filters per site and two replicates per filter).

Reactions were performed in a 25 µl final volume: 12.5 µl of TaqMan Environmental Master Mix® 2.0 (Life Technologies, Applied Biosystems), 2.5 µl of each primer (5 µM), 1 µl of probe (5 µM), 1.5 µl of sterile water and 5 µl of eDNA. Each 96-well plate contained four control samples (qPCR mix to which water was added instead of eDNA) to test for possible contamination during amplification. *In-situ* negative controls (distilled water filtered between each site) were analysed in duplicate.

Standard curves generated by performing serial dilutions with DNA extracted directly from individuals collected in Martinique were also included to assess the quality of the amplified products. The concentrations of the dilutions ranged from 20 ng µl<sup>-1</sup> (NanoDrop 1000 spectrophotometer) to 2.375 × 10<sup>-7</sup> ng µl<sup>-1</sup>. A signal was considered positive when the number of amplification cycles (*Ct* value) did not exceed 42 (Agersnap et al., 2017) and the site was considered to harbour *A. cryptocallus* if at least one replicate out of six tested was positive (Bedwell & Goldberg, 2020).

## 2.6 | Limit of detection and limit of quantification

To investigate the sensitivity of the qPCR method, the requirements in the Minimum Information for Publication of Quantitative Real-Time PCR Experiments guidelines defined by Bustin et al. (2009) were followed and both the limit of detection (LOD) and the limit of quantification (LOQ) were determined. The LOD corresponded to the minimum concentration at which eDNA could be detected and the

LOQ corresponded to the minimum concentration at which quantification was possible. The LOD and LOQ were determined following the analysis of the previously described dilution series, with concentrations ranging from 20 to 2.4 × 10<sup>-7</sup> ng µl<sup>-1</sup>, where each dilution was analysed in 10 replicates. A modelling of the results, following Klymus et al. (2019), was carried out with slight modifications as DNA concentrations were measured in ng µl<sup>-1</sup>. The model was performed with the following parameters: the 'Best' fitting model in the *LOD.FCT* and *LOQ.FCT* functions, and 0.7 for the *LOQ.threshold* function.

## 2.7 | Inhibition test

To test the level of inhibition in eDNA samples, DNA from a species not occurring in Martinique, the white-clawed crayfish (*Austropotamobius pallipes*), was used. The specific habitat requirement of this rare and endangered European species makes it unable to be present and survive in a Martinique freshwater system. In addition, species-specific primers and probes have already been developed and thoroughly tested for this species (Troth et al., 2020). An inhibition test was conducted by 'spiking' aliquots of eDNA samples with a low concentration of DNA extracted from the white-clawed crayfish to assess the presence of inhibitors. 'Clean' control samples consisting of ddH<sub>2</sub>O were spiked as a reference of samples with no inhibition. Control reactions were performed in mixes containing 12.5 µl of TaqMan Environmental Master Mix 2.0 (Life Technologies, Applied Biosystems), 2.5 µl of each white-clawed specific primer (5 µM), 1 µl of white-clawed specific probe (5 µM), 1 µl of white-clawed DNA (known concentration of 4 ng µl<sup>-1</sup>) and 5.5 µl of DNA-free water. Subsequently, the test reactions were performed as follows: 12.5 µl of TaqMan Environmental Master Mix 2.0 (Life Technologies, Applied Biosystems), 2.5 µl of each white-clawed specific primer (5 µM), 1 µl of white-clawed specific probe (5 µM), 1 µl of white-clawed DNA (known concentration of 4 ng µl<sup>-1</sup>), 4 µl of eDNA and 1.5 µl of DNA-free water. Each filter (biological replicate, referred to as 'eDNA' immediately above) used in this study was tested and the average cycle number was calculated for each station. The inhibition level was then calculated for each station by subtracting the average number of test cycles from the average number of control cycles. Inhibition can be characterized by a delay of more than one cycle ( $\Delta Ct > 1$ ). Control samples (qPCR mix to which water was added instead of DNA/eDNA) were also added in the plate, to test for possible contamination during amplification as well as to act as non-inhibited samples.

## 2.8 | eDNA transport

The effectiveness of the method, in terms of the distance at which eDNA can be detected in the main river, was tested through an 'eDNA transport' experiment. For this, the Coeur Bouliki area was chosen, where *A. cryptocallus* was known to be present in a ditch with

a very weak flow rate, connected approximately 20 m downstream to the main river (called Rivière Blanche). Water was therefore filtered (as described above) directly from the ditch and then downstream from the river bed at four different points (200, 500, 1,000 and 1,500 m; Figure 2). One biological sample (filter) was collected per site and then two technical replicates were performed per filter. To ensure that the DNA detected was from the targeted fish population, a complete inventory of the ditches and backwaters present in the study area was carried out using a dip net.

## 2.9 | Analysis

The maps were generated using QGIS 2.18 (Las Palmas) software (QGIS Development Team, 2016), with the Martinique map coming from the IGN database and the watercourses from BD Carthage and BD Topo. All field data (sites, coordinates, altitude, stream, total volume filtered and the results from traditional sampling methods) and laboratory results (proportion of qPCR positive replicates, mean Ct values  $\pm$  SD) are presented in the Supplementary Information (Appendix B).

RStudio v1.1.463 (R Development Core Team, 2019) was used to perform statistical analyses. The Shapiro–Wilk normality test was used to verify the normal distribution of the data and site occupancy modelling approaches to assess the effects of ‘total volume filtered’ on the ‘probability of *A. cryptocallus* eDNA occurrence’ were run (MacKenzie et al., 2002; Royle & Dorazio, 2008). The ‘eDNAoccupancy’ package was used following procedures given in Dorazio & Erickson (2018). The ‘occModel’ function from the described package fitted the model, and Markov Chain Monte Carlo (MCMC) were run for 10,000 iterations to obtain parameter estimates and credible intervals.

The probabilities of false-positive and false-negative errors (in the field and in the laboratory) were estimated, using the model

developed by Griffin et al. (2020), under the default settings, on the platform (<https://seak.shinyapps.io/eDNA/>). The detection or absence of *A. cryptocallus* was put as a covariate. The probability of species presence ( $\psi$ ), the probability of eDNA presence given species presence ( $\theta_{11}$  or true positive field sample), the probability of eDNA presence given species absence ( $\theta_{10}$  or false positive field sample), the probability of eDNA detection given eDNA presence ( $p_{11}$  or true positive qPCR detection), and the probability of eDNA detection given eDNA absence ( $p_{10}$  or false positive qPCR detection) were investigated. The results are reported in Table 2.

## 3 | RESULTS

### 3.1 | qPCR assays

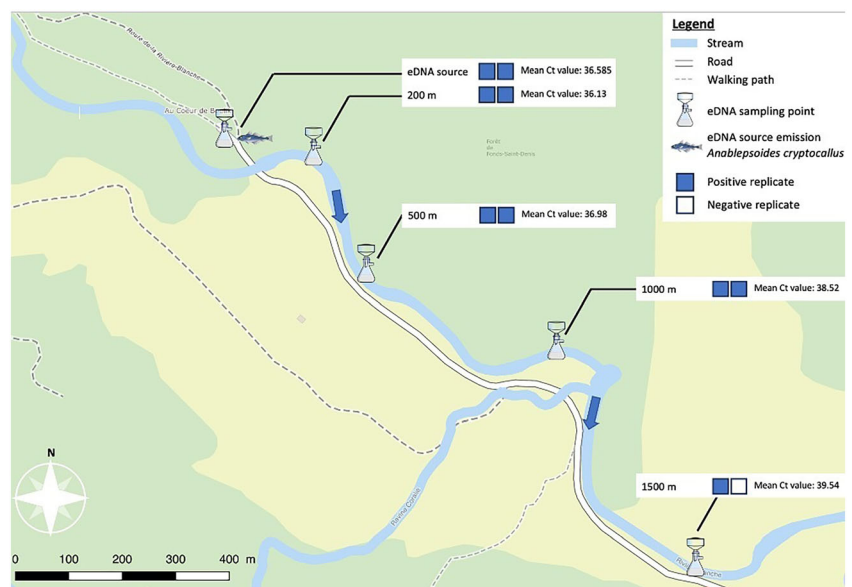
No matches occurred with other species during *in-silico* alignment tests using online databases (Supplementary Information; Appendix D). An analysis of the results shows that the optimal conditions for sample processing were 5  $\mu$ M of primers and probe concentration and 60°C for the annealing temperature. Under these conditions, primers/probe give optimal results for *A. cryptocallus* detection, and no amplification of other DNA samples tested *in-vitro* (*H. plecostomus*, *H. robinii*, *P. reticulata*, *Ancystrus* sp, *K. marmoratus*) occurred.

The LOD corresponded to a concentration of  $2.6 \times 10^{-5}$  ng  $\mu$ l<sup>-1</sup> and the LOQ corresponded to a concentration of  $8.1 \times 10^{-5}$  ng  $\mu$ l<sup>-1</sup> ( $P$ -value < 0.001).

### 3.2 | Mesocosm

All eDNA samples from the controlled mesocosms (including both volume conditions) showed positive detection of *A. cryptocallus*

**FIGURE 2** Evaluation of eDNA dispersal along the Rivière Blanche from a known population of *Anablepsoides cryptocallus*. Blue squares represent positive replicates for the presence of *A. cryptocallus*, and white squares negative replicates. The Average Ct value represents the number of cycles used for the detection of the *A. cryptocallus* target DNA.



by real-time PCR. This was observed after an average of  $20.57 \pm 0.19$  cycles for the 1.5 L condition and after an average of  $32.105 \pm 0.23$  cycles for the 50 L condition. An influence of the volume of the mesocosm on the detection efficiency was observed, with a decrease in the number of cycles when the mesocosm volume decreased.

### 3.3 | eDNA transport and inhibition test

Detection of *A. cryptocallus* DNA was possible at each sampling point up to 1,500 m (Figure 2), with decreasing efficiency the further away from the ditch, in the principal river: from an average of  $36.13 \pm 0.1$  cycles at 200 m from the source of eDNA emission to 39.54 cycles at 1,500 m. Both technical replicates were positive at each point, except for the furthest point (1,500 m, only one replicate out of two) (Figure 2).

The maximum inhibition level ( $\Delta Ct$ ) observed in eDNA field samples was  $0.3 \pm 0.07$  for the Ranch Macabou station. Most of the stations had an  $\Delta Ct$  value  $< 0.1$ , confirming that inhibition did not affect the detection probability of *A. cryptocallus* eDNA.

### 3.4 | In-situ detection

No negative samples tested positive, indicating the absence of contamination between sites or during laboratory procedures. Of the 57 sites surveyed using eDNA analysis, 27 were found to be positive, with an amplification rate between one and six out of the six technical replicates, corresponding to 19 different rivers and two wetlands (Table 1, Figure 3). The mean Ct values for these positive stations ranged from  $34.045 \pm 0.44$ , with a proportion of qPCR positive replicates reaching 100% (for Golf Trois Ilets), to 41.2 for the Balheu station, with only 1/6 (17%) qPCR positive

**TABLE 1** Sites where *Anablepsoides cryptocallus* was detected by qPCR from eDNA, including total filtered volume, the proportion of qPCR positive replicates and the mean Ct value ( $\pm$  standard deviation), as well as detection using standard kick sampling. Stations highlighted with a '+' in the last column are those where the presence of *A. cryptocallus* has been validated in 2019 by Biotope (2020)

Sites	Total volume filtered (ml)	Proportion of qPCR positive replicates	Mean Ct value ( $\pm$ SD)	Traditional method detection	Biotope*
Pont de Bassignac	1,150	0.833	37.2 ( $\pm$ 1.97)	–	
Fonds Galion – Sainte Luce	2,400	1	36.38 ( $\pm$ 1.07)	–	
Usine du Galion	600	1	35.86 ( $\pm$ 1.29)	+	
Golf Trois Ilets	2,200	1	34.045 ( $\pm$ 0.44)	+	+
Petit Bourg	1,600	0.17	38.73 ( $\pm$ 0)	–	
Parcours Sportif Saint-Esprit	1,550	0.33	38.26 ( $\pm$ 0.83)	–	
AMEP Moutte	2,400	0.67	40.275 ( $\pm$ 0.51)	–	
Balheu	850	0.17	41.2 ( $\pm$ 0)	–	
Pont N8	1,000	0.67	38.27 ( $\pm$ 2.36)	+	+
Quartier Rivière Lézarde 2	3,800	1	40.03 ( $\pm$ 0.32)	+	
Quartier Hotel Plaisir	1,400	0.17	40.96 ( $\pm$ 0)	–	
Rivière Caleçon	1,700	0.83	37.82 ( $\pm$ 0.33)	–	
Denel	1,800	0.67	38.5 ( $\pm$ 1.35)	–	
Marigot	1,150	0.17	39.75 ( $\pm$ 0)	–	
Seguineau	2,900	0.5	38.61 ( $\pm$ 1.72)	–	
Ravine Lorrain	1,700	0.5	40.23 ( $\pm$ 1.43)	–	
Morne Vallon	1,250	0.67	39.05 ( $\pm$ 0.7)	–	
Cœur Bouliki	2,650	1	36.68 ( $\pm$ 0.47)	+	+
Fond Labour	2,800	0.17	39.81 ( $\pm$ 0)	–	
Fonds Saint-Jacques	2,700	0.17	38.23 ( $\pm$ 0)	–	
EPLFPA Robert	1,500	0.33	40.125 ( $\pm$ 0.5)	–	
Grands Fonds – François	1,500	0.67	39.12 ( $\pm$ 0.73)	–	
Rue Jambette	750	0.33	40.015 ( $\pm$ 0.19)	–	
Tivoli	800	1	37.61 ( $\pm$ 0.32)	+	+
Petit Pré	900	1	35.05 ( $\pm$ 0.53)	+	+
François Jetée	300	0.67	39.67 ( $\pm$ 0.91)	+	+
Anses d'Arlets	300	1	36.78 ( $\pm$ 0.13)	+	+

replicates (Table 1, Figure 3). Of the positive sites surveyed, *A. cryptocallus* had already been captured at seven sites by conventional fishing during the study conducted by Biotope (2020): Golf Trois Ilets – 6/6 replicates (Ct value  $34.045 \pm 0.44$ ); Pont N8 – 4/6 replicates (Ct value  $38.27 \pm 2.36$ ); Coeur Bouliki – 6/6 replicates (Ct value  $36.68 \pm 0.47$ ); Tivoli – 6/6 replicates (Ct value  $37.61 \pm 0.32$ ); Petit Pré – 6/6 replicates (Ct value  $35.05 \pm 0.53$ ); François – 4/6 replicates (Ct value  $39.67 \pm 0.91$ ); and Anses d'Arlets – 6/6 replicates (Ct value:  $36.78 \pm 0.13$ ) (Table 1, Figure 3). Some catchments appear to have several sites containing *A. cryptocallus* – for example, Lézarde, Galion, Coulisses, François, Longvilliers and Lorrain (Figure 3). No populations were recorded in the North Atlantic coast area, North Caribbean or in the South Atlantic area.

During the dip net surveys, nine sites were found to be positive, but no site was found to be positive by this monitoring method alone (i.e. these nine sites were also positive using eDNA detection). All of the seven positive sites historically recorded in 2019 were investigated, and two new points of presence were discovered: Usine du Galion and Quartier Rivière Lézarde 2. Among the 27 positive sites, 18 have not yet been confirmed by fishing, and therefore require additional field survey to confirm the positive eDNA detection (Table 1).

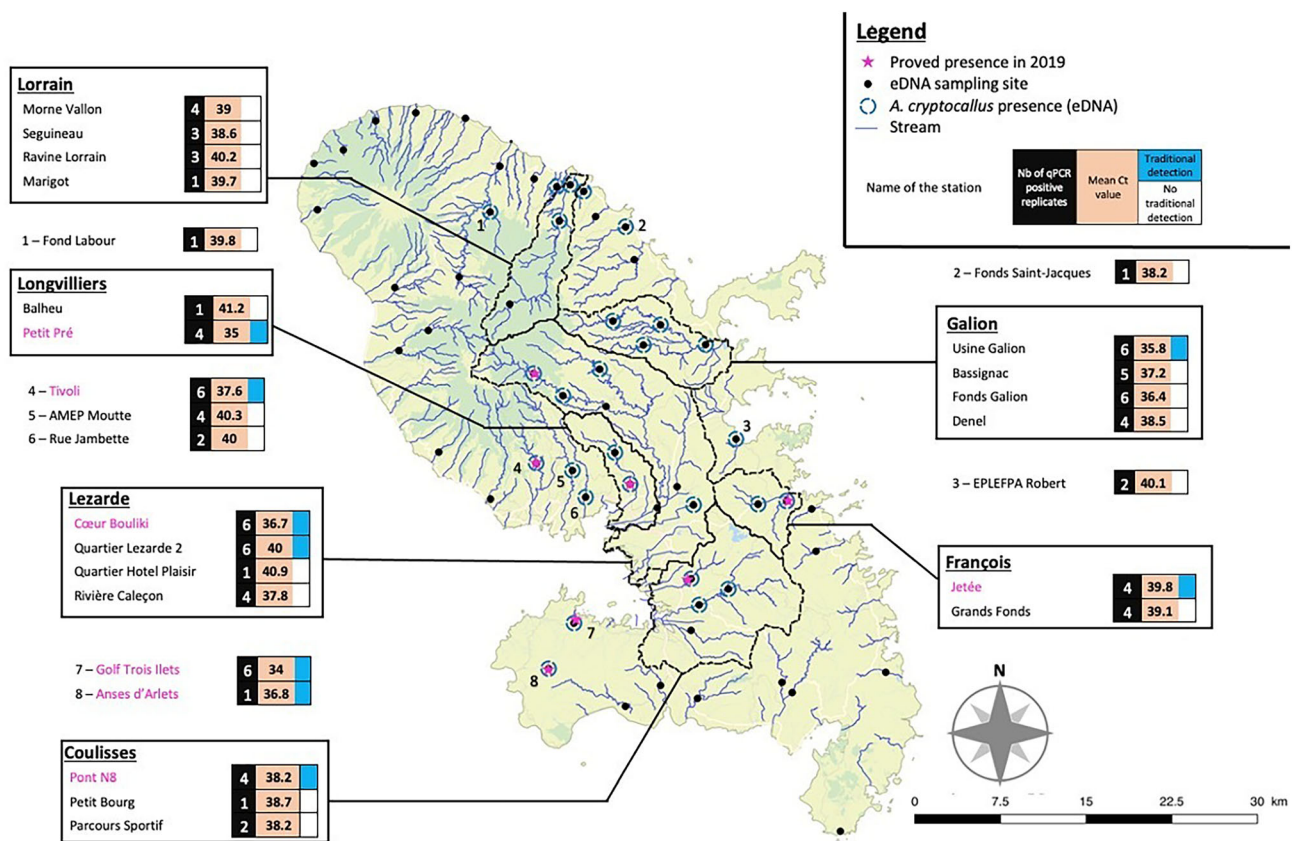
### 3.5 | Occupancy modelling and false negative/positive errors

Occupancy modelling showed a significant effect of total volume filtered per station on the probability of *A. cryptocallus* eDNA occurrence ( $\text{adj-}R^2 = 0.977$ ;  $P\text{-value} < 0.001$ ; Figure 4). The optimal probability of occurrence occurred between 300 and 1,000 ml of water filtered, and the probability of occurrence then decreased up to 50% of occurrence probability for 6,000 ml filtered and around 25% for 7,500 ml.

Posterior mean values of *A. cryptocallus* presence probability  $\Psi$  (0.449) and the probability of true positive field sample  $\theta_{11}$  were in accordance with expected values of the model (respectively 0.5 and 0.88) (Table 2). The probability of true positive qPCR detection  $p_{11}$  was a little lower than expected value ( $p_{11}$ : 0.738 vs 0.9). The model returned very low probabilities of false positive field sample  $\theta_{10}$  (0.01) and false positive qPCR detection  $p_{10}$  (0.005).

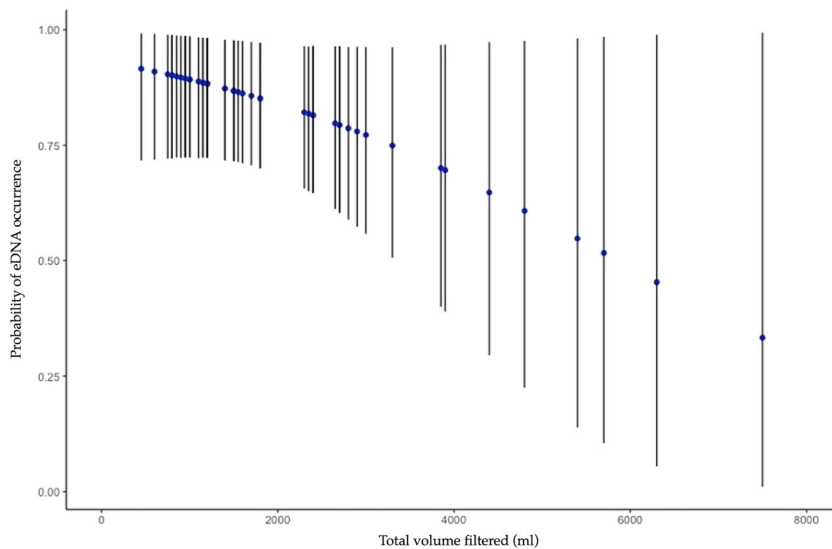
## 4 | DISCUSSION

eDNA detection for monitoring *A. cryptocallus* was found to be accurate and reliable in establishing a fast and exhaustive mapping of



**FIGURE 3** Distribution map of *Anablepsoides cryptocallus* in the rivers of Martinique based on detection by eDNA. The sampling was based on known data on the presence of *A. cryptocallus* (pink stars) and data from the Biotope presence model (Supplementary Information; Appendix A).





**FIGURE 4** Influence of total volume filtered on the probability of *Anablepsoides cryptocallus* eDNA occurrence, following site occupancy modelling.

**TABLE 2** False negative and false positive rates in eDNA-based detection, following the modelling approach of Griffin et al. (2020) (<https://seak.shinyapps.io/eDNA/>)

	2.5 Credible Interval	Posterior Mean	97.5 Credible Interval	Expected Value
$\psi$ : Probability of species presence	0.313	0.449	0.594	0.5
$\theta_{11}$ : Probability of true positive field sample	0.674	0.889	0.995	0.88
$\theta_{10}$ : Probability of false positive field sample	$5.213 \times 10^{-5}$	0.012	0.062	0.11
$p_{11}$ : Probability of true positive qPCR detection	0.584	0.738	0.803	0.9
$p_{10}$ : Probability of false positive qPCR detection	$3.245 \times 10^{-5}$	0.005	0.026	0.1

the distribution of this endemic species in Martinique. Indeed, the validation steps recommended by Thalinger et al. (2021) have shown the specificity of the primers and the probe *in-silico* and *in-vitro*. The LOD and LOQ (respectively  $9.5 \times 10^{-7}$  and  $1.5 \times 10^{-5}$  ng  $\mu\text{l}^{-1}$ ) showed a highly sensitive detection method. Extracted genomic DNA was used, instead of a gBlock, to establish the LOD and LOQ. As in some of the other recent studies [marine pelagic fish species in Wong et al. (2022) and *Esox lucius* in Karlsson et al. (2022)] which used genomic DNA for the LOD and LOQ calculations, we assumed no interference during PCR from the rest of the species' genome, other genetic material or potential co-extracted inhibitory compounds. The mesocosm experiments, as well as sites where *A. cryptocallus* had been captured previously and which served as positive controls, allowed further validation of the methodology. Statistical modelling provided the means to investigate the effects of field parameters (total filtered volume) on the detection efficiency of this cryptic fish species, and to estimate the probabilities of false negatives or false positive errors.

Of the 57 sites surveyed, 27 were found to be positive for the presence of *A. cryptocallus*, which is a significant increase over the Biotope study that identified the species in 10 sites only by traditional fishing. In the present study, only seven of the 10 sites fished by Biotope were retained owing to adverse weather conditions (notably drought), which made eDNA surveys unfeasible. These sites were all positive using the molecular survey method, with high detection rates

(Table 1, Figure 3). Among the areas historically known to contain this species, the Galion area was found to be positive (using both eDNA and fishing), although no fish had been caught during the field surveys carried out by Biotope (2020). This result reflects the high efficiency of this eDNA detection method compared with more invasive traditional inventory methods, already reported in the literature, such as for the redclaw crayfish *Cherax quadricarinatus* (Baudry et al., 2021).

Overall, of the 27 eDNA positive sites, nine were confirmed by dip netting (33.3%) (Table 1). This low percentage of confirmation, compared with other studies conducted in Martinique, can be explained by the following points:

- (1) The difficulty of capturing individuals using traditional fishing techniques – *A. cryptocallus* lives in muddy ditches or even in areas often disconnected from the main river, and these areas are hard to survey effectively. In addition, during the dry season, when water is scarce, *A. cryptocallus* can bury itself in the mud for several days or even weeks, making it invisible and hard to capture. The capture of several individuals at nightfall could also suggest a nocturnal life rhythm. However, most surveys in this study were carried out during the day.
- (2) The low density of fish makes its capture difficult and more random. This may be reflected by the low number of positive technical replicates at specific sites when small amounts of little

eDNA are released into the environment. Moreover, for the sites showing 6/6 replicate eDNA positives, most were confirmed by fishing (five out of six sites).

- (3) The distance of the population from the source of eDNA emission – indeed, the eDNA detection experiment revealed possible detection up to 1,500 m in the main river. Thus, the presence of an upstream population could be detected at several downstream sites in the same river. A reduction in detection efficiency was observed from upstream to downstream, with a decrease in the number of positive technical replicates. However, this may also be explained by the presence of fish in a larger body of water, and therefore with greater dilution of the DNA, but as large rivers are not the preferred habitat for *A. cryptocallus*, upstream presence seems the most likely explanation.

Several rivers illustrate these results well: the Rivière Couliesses with the upstream site Parcours sportif Saint-Esprit (2/6 replicates) and the downstream site Petit Bourg (1/6 replicates), Rivière Blanche with the upstream site Cœur Bouliki (6/6 replicates) and the downstream site Quartier Hotel Plaisir (1/6 replicates) and the Rivière Galion with the upstream site Saint-Luce (6/6 replicates) and the downstream site Bassignac (5/6 replicates). Some rivers were not sampled in the upstream part, but showed positive eDNA results in their downstream part: for example, the Saint-Jacques River (1/6 replicates), the Cacao River (site EPLEFPA Robert with 2/6 replicates) and the Jambette River (site Rue Jambette with 2/6 replicates). Thus, it is highly likely that the presence of *A. cryptocallus* is located further upstream from these sites and therefore not recorded by traditional methods at the sites sampled. It is important to note that this technique makes it possible to point to novel survey sectors upstream from the sampling sites, and positive detection could be an indication to increase sampling upstream, rather than an actual point of presence.

The influence of abiotic parameters (here, the total volume filtered per station) on the efficiency of detection of *A. cryptocallus* was investigated from water samples in the main river bed. Interestingly, the results demonstrated a decrease in the probability of fish detection as the filtered volume increases (Figure 4). This contrasts with other studies, demonstrating an increase in detection efficiency when large volumes are filtered (Cantera et al., 2019; Strand et al., 2019). Filtering small quantities of water is not recommended if the aim is to increase the probability of capturing eDNA. Indeed, increasing the filtration effort leads to an increase in the amount of DNA captured (targeted and non-targeted). These results can probably be explained by the ecological preferences of *A. cryptocallus*: this species of fish is associated particularly with turbid and lentic areas, highly loaded with organic matter, where filtration volumes are lower. The modelling method of Griffin et al. (2020) was used to tackle the issues around potential false negatives and false positives. The study was carried out using three biological replicates (on-site filtrations) and two technical replicates (qPCR tests in the laboratory), for a total of six technical replicates per station. Baudry et al. (2021) demonstrated a near 100% detection efficiency of the target species (here, the red claw crayfish *C. quadricarinatus*) from

three biological replicates and similar results were found by Mauvisseau et al. (2019). However, the results demonstrated a high reliability of the method with both a low probability of false positives and false negatives in the field and the laboratory (Table 2). The presence of historical DNA in the sediments could be the source of false positives, where a species that was once present has since disappeared. However, this scenario seems very unlikely given that eDNA is only known to persist in the water column for a few days to several weeks (Minamoto et al., 2012; Li et al., 2019), with even greater degradation in tropical areas where UV and temperature are very strong (Baudry et al., 2021). Moreover, the rainy episodes, known to be intense in the tropical areas, induce a sweeping of the sediments and a physical degradation (until the elimination) of the eDNA retained in them. Nevertheless, the eRNA-based monitoring technique in future studies could be used to investigate this persistence, since eRNA is expected to degrade more rapidly than eDNA (Cristescu, 2019; Wood et al., 2020).

The presence of *A. cryptocallus* in some catchment areas, such as the Lézarde, Galion, Couliesses and Lorrain, is very interesting. For example, the discovery of positive records in certain North Atlantic sites is encouraging because they are among the areas potentially favourable to the presence of *A. cryptocallus* (Supplementary information; Appendix A), even though the last surveys in 2019 were unsuccessful (Biotopie, 2020). This study has demonstrated the need to carry out conventional fishing to confirm the presence of this species in the positive sites identified. This is especially important at upstream sites, as it will then be possible to refine the precise location of the populations by taking additional samples in the upstream part when sites located downstream have proved positive. These surveys should be carried out during the wet season to avoid the individuals burying themselves in the mud. Despite the discovery of new populations, the abundance of these fish in Martinique remains low and with a declining area of occurrence. Their rarity mainly results from the modification, even the destruction, of their ecological niche. This species lives in wetlands and ditches, and these are intensely targeted by human activities (cleaning, drainage, drying out, etc.) that lead to habitat destruction, loss and fragmentation.

#### 4.1 | Implications for conservation

These ecosystems, especially wetlands or ditches, receive less attention than other habitats and protection programmes are therefore difficult to establish (Flitcroft et al., 2019). The presence of this rare endemic species in these ecologically important areas should promote a better protection of these habitats and benefit other co-occurring species. However, despite its recognized status as the only endemic freshwater fish species in Martinique (Lim et al., 2002), *A. cryptocallus* still does not benefit from any particular protection status. The present study makes an important contribution to the baseline data for an IUCN Red List of Threatened Species assessment for this taxon, and this in turn can help to prioritize it for targeted conservation efforts. It would be very valuable to study the health

status of the populations as well as their genetic diversity (using microsatellite methods) to establish management units and conservation priorities, which could also lead to a protection status for *A. cryptocallosus*. In addition, this study shows the importance of using eDNA methods in the conservation of endangered species, as well as demonstrating progress in tropical regions (and more precisely here of Martinique) in the use of this molecular method. These tropical areas, although considered as hotspots of biodiversity, sheltering a rich and very fragile aquatic fauna, are little studied, resulting in an under-representation in the literature. For example, 90% of eDNA studies have been carried out in temperate zones, and in particular in North America (52%), Europe (20%) and Asia (19%) (Belle, Stoeckle & Geist, 2019). The majority of eDNA studies referenced since 2019, in the tropics, were carried out in Australia and South Asia (Belle, Stoeckle & Geist, 2019). This study is therefore of paramount importance, both in the progress it represents in the use of eDNA in the tropics and its application to a species, strictly endemic to Martinique, of major ecological and territorial importance. Despite all the challenges that these tropical environments impose (e.g. water temperature and DNA degradation), the eDNA method can now be applied efficiently in tropical environments, which will be of great use in the field of conservation.

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#### CONFLICTS OF INTEREST

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

#### AUTHOR CONTRIBUTIONS

**Thomas Baudry:** Conceptualization; methodology; software; formal analysis; field investigation; data curation; writing – original draft; writing – review and editing; visualization; validation; project administration; funding acquisition. **Quentin Mauvisseau:** Conceptualization; methodology; software; writing – review and editing; visualization. **Alexandre Arqué:** Supervision; field investigation; writing – review and editing; project administration; funding acquisition. **Jean-Pierre Goût:** Supervision; field investigation; writing – review and editing; project administration. **Carine Delaunay:** Methodology; validation. **Hugo J. de Boer:** Writing – review and editing. **Frédéric Grandjean:** Conceptualization; writing – review and editing; supervision; project administration; funding acquisition.

#### DATA AVAILABILITY STATEMENT

All data generated or analysed 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, on reasonable request.

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## SUPPORTING INFORMATION

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