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Detection of a global aquatic invasive amphibian, *Xenopus laevis*, using environmental DNA

Jean Secondi[1,2,*], Tony Dejean[3], Alice Valentini[3], Benjamin Audebaud[4], Claude Miaud[5]

Abstract. Detection is crucial in the study and control of invasive species but it may be limited by methodological issues. In amphibians, classical survey techniques exhibit variable detection probability depending on species and are often constrained by climatic conditions often requiring several site visits. Furthermore, detection may be reduced at low density because probability capture (passive traps), or activity (acoustic surveys) drop. Such limits may impair the study of invasive species because low density is typical of the onset of colonisation on a site. In the last few years, environmental DNA (eDNA) methods have proved their ability to detect the presence of aquatic species. We developed here an eDNA method to detect *Xenopus laevis* in ponds. This austral African species is now present worldwide because of its use in biology and as a pet. Populations have settled and expanded on several continents so that it is now considered as one of the major invasive amphibians in the World. We detected the presence of *X. laevis* at density as low as 1 ind/100 m^2 and found a positive relationship between density in ponds and rate of DNA amplification. Results show that eDNA can be successfully applied to survey invasive populations of *X. laevis* even at low density in order to confirm suspected cases of introduction, delimit the expansion of a colonized range, or monitor the efficiency of a control program.

Keywords: African clawed frog, biological invasion, density, detection method, invasive species.

Biological invasions are considered to be a major threat to most ecosystems (Lockwood et al., 2012). Like many other groups amphibians and reptiles have been experiencing declines or extinctions after the introduction of alien species (Martin and Murray, 2010; Ficetola et al., 2011). They also provide their share of successful biological invaders, among which the Cane toad *Rhinella marina*, the bullfrog *Lithobates catesbeianus*, or the brown tree snake *Boiga irregularis* are known for their strong impact on local communities (Li et al., 2011). These species affect ecological networks because of their large size and the lack of adaptation of the local fauna (Shine, 2010). Reliable detection of such species is a major issue in invasion biology to identify colonized sites, delimit colonized ranges, or ascertain eradication success (Morrison et al., 2007). Meeting these objectives is challenging because population density is often low at the onset of settlement or after an intensive control program. Caudata are usually caught by passive traps or dip nets whereas acoustic surveys are often used for anurans (Heyer et al., 1994). At low density, these survey techniques may exhibit low detection probability because of dilution or reduced behavioural activity. To circumvent this problem, increased survey intensity may be required but costs increase with the surveyed area and the number of site visits (Lockwood et al., 2012). This means that the detection step may fail because of methodological or funding issues. Owing to increasing introduction rate of invasive populations, the development of highly informative and cost-efficient survey methods becomes a priority to detect early alien species and map their ranges. Environmental DNA (eDNA) is a promising approach in this regard. This technique is well-suited for freshwater invertebrates and vertebrates (Ficetola et al., 2008; Lodge et al., 2012; Thomsen et al., 2012) because DNA remains available up to a month in

1 - GECCO, Université d'Angers, Angers, France
2 - UMR CNRS 6554 LETG – LEESA, Université d'Angers, Angers, France
3 - Spygen, Le Bourget du Lac, France
4 - Agglomération du Bocage Bressuirais, Bressuire, France
5 - EPHE, UMR 5175 CEFE, Montpellier, France
*Corresponding author; e-mail: jean.secondi@univ-angers.fr

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the medium (Dejean et al., 2011), and only one sample is required, which drastically reduces time constraint on sampling. Furthermore, detection rates are often higher for eDNA than for classical surveys (Pilliod et al. (2013), but see Thomsen et al. (2012)), even for large and loud species such as the American Bullfrog (Dejean et al., 2012).

We report here the successful use of the eDNA technique to detect the presence of another global invasive amphibian species, the African clawed frog (*Xenopus laevis*). For decades, the species has been exported from South Africa to many parts of the World, originally for pregnancy testing, and was later used as a model organism for biology (Weldon et al., 2007). The species is also available for sale as a pet in many countries (Measey et al., 2012). Consequently, established populations have been reported in North America, South America, Europe, and Asia, several of which have thrived and expanded so much that they are now considered as invasive (Measey et al., 2012). *X. laevis* is a large-bodied species that is tolerant to a broad range of environmental conditions (Measey et al., 2012). It is also a generalist predator of vertebrate and invertebrate communities in lentic environments (Measey, 1998; Faraone et al., 2008; Amaral and Rebelo, 2012). *X. laevis* can negatively affect local amphibian communities (Lillo et al., 2011) and impact on invertebrate are expected because it exploits resources from ponds year round (Measey, 1998). Finally, *X. laevis* is an asymptomatic carrier of the chytrid fungus and a carrier of ranavirus (Robert et al., 2007; Ouellet et al., 2012). These pathogens are responsible for events of mass mortality in amphibians worldwide (Briggs et al., 2005; Bosch and Martinez-Solano, 2006). The global distribution of the species due to trade, its propensity to spread in various environments, its potential role as a pathogen reservoir, and its impact on local communities make *X. laevis* one of the major invasive amphibians in the world.

Xenopus laevis is aquatic at all stages. Adults of both sexes call in water (Tobias et al., 1998) but their soft signals are difficult to detect. Therefore, the species is surveyed using funnel traps and several site visits are necessary to ascertain presence at low density. For these reasons, the time lag between introduction and detection is often long, up to 25 years (Measey et al., 2012). *X. laevis* has been released in Western France in the early 80's, probably after a breeding facility has been disabled, but the population was not detected before the early 2000's (Fouquet and Measey, 2006). The colonized range is now over 500 km^2 and it may shortly reach wetlands of international importance (Brenne, Sologne). Given the fast expansion and the area now covered by the species (Fouquet and Measey, 2006), classical survey methods may prove logistically unsustainable. We therefore developed an eDNA protocol and tested its ability to detect the species presence at several densities in ponds.

Sampling strategy and estimation of density. Following the sampling strategy by Tréguier et al. (2014), we surveyed 9 cattle ponds (area 90-1500 m^2). Six ponds were within the colonized range in France and displayed different density levels. Pond selection was carried out by analysis of capture records from previous years. The last three ponds were about 150 km away from the colonized range. For all ponds, we collected 20 water samples of 40 ml along a transect on July 17th 2013. Transects were approximately 100-150 m long and parallel to the shore. Samples were evenly distributed along each transect, and always taken from areas where water column was less than 1 m deep. Samples from the same pond were pooled into a common sterile plastic bag and mixed. Six subsamples of 15 ml were immediately taken and added in 6 tubes (50 ml) filled with a solution of 1.5 ml of sodium acetate 3 M and 33 ml of absolute ethanol. Tubes were stored at $-20°C$ until DNA extraction.

We estimated the abundance of *X. laevis* in ponds after eDNA sampling by using standard

fish funnel traps (diameter 40 cm; length 70 cm; entrance diameter 15 cm) in 3 site visits over 3 consecutive days from 20-23 August 2013. Caught individuals were euthanasied by freezing as stated in the authorization. The number of traps was adjusted to the area of the pond giving a sampling effort of 0.005 trap/m^2 to 0.022 trap/m^2. We standardised the number of catches by the sampling effort as follows: $C = N/(A/N_t)$ where C is the relative abundance, N the number of individuals caught over the three site visits, A pond area, and N_t trap number. We used generalized linear models to test the relationship between standardised abundance and detection rate in water samples. Analyses were carried out with R 3.0 (R Core Team, 2014).

Environmental DNA analysis. DNA sequences from the cytochrome b, the COI, 12S and 16S genes for *X. laevis* and closely related species were retrieved from GenBank®. The 12S region was identified as the best candidate for designing the assay. eDNA samples were analysed using primers and probes designed especially for this study using Geneious 6 (Biomatters, available at http://www.geneious.com/) to amplify a 83-bp fragment (primer included) of the 12S region (SPY_XenLea_F 5′-AGGCT TAATGATTTTGCATC-3′, SPY_XenLea_R 5′-AGGGTATAGAAAATGTAGCC-3′ and SPY_ XenLae_Probe 5′-FAM-ACGTCAGGTCAAG GTGTAGCA-BHQ1. The *in silico* analysis was performed with an electronic PCR using ecoPCR software (Ficetola et al., 2010), available at https://git.metabarcoding.org/obitools/ecopcr/wikis/home) on the EMBL-Bank release 114 (released in December 2012) and the SPYGEN reference database which includes 56 European amphibian species. Those primers amplify 12 species (*Tragulus javanicus*, *X. tropicalis*, *X. andrei*, *X. boumbaensis*, *X.* cf. *boumbaensis* BJE-2007, *X.* cf. *fraseri* 2 BJE-2004, *X. gilli*, *X. laevis*, *X. largeni*, *X. longipes*, *X. petersii*, *X. victorianus*), among which only *X. laevis* is present in France.

DNA extraction was performed as in Treguier et al. (2014). The six subsamples per site were centrifuged at 14 000× *g*, 30 min, 6°C and the supernatant was discarded. After this step, 360 μl of ATL Buffer of the DNeasy Blood & Tissue Extraction Kit (Qiagen) were added in the first tube, the tube was vortexed and the supernatant was transferred to the second tube. This operation was repeated for all the six tubes. The supernatant in the 6th tube was transferred in a 2 ml tube and the DNA extraction was performed following the manufacturer's instructions. DNA extraction from water was performed in a dedicated room equipped with positive air pressure, UV treatment and frequent air renewal. Laboratory personnel wear full protective clothing,which is put on before entering the extraction room. Extraction of negative controls was performed to monitor possible contamination. qPCR was performed in a final volume of 25 μl, which included 3 μl of template DNA, 12.5 μl of TaqMan® Environmental Master Mix 2.0 (Life Technologies®), 6.5 μl of ddH2O, 1 μl of each primer (10 μM) and 1 μl of probe (2.5 μM). Each sample was run in 12 replicates. The tubes containing the eDNA samples were then sealed, qPCR standards were added to the qPCR plate in a separate room from the eDNA extraction room. A dilution series of *X. laevis* DNA (10^{-1}-10^{-4} ng μl^{-1}) was used as a qPCR standard (two replicates per concentration). Four negative (ddH2O water) controls were added during the qPCR step. qPCR runs were performed in a third room, dedicated to amplified DNA analysis with negative air pressure and physically separated from the eDNA extraction room. Samples were run on a BIO-RAD® CFX96 Touch real time PCR detection system, under thermal cycling at 50°C for 5 minutes and 95°C for 10 minutes, followed by 55 cycles of 95°C for 30 seconds and 51.3°C for 1 minute. This annealing temperature was estimated using a gradient PCR. eDNA detection rate was calculated as the number of positive qPCR amplifications (qPCR replicates) over the total number of qPCR replicates.

Results and discussion. We detected no false positives in water samples from ponds out-

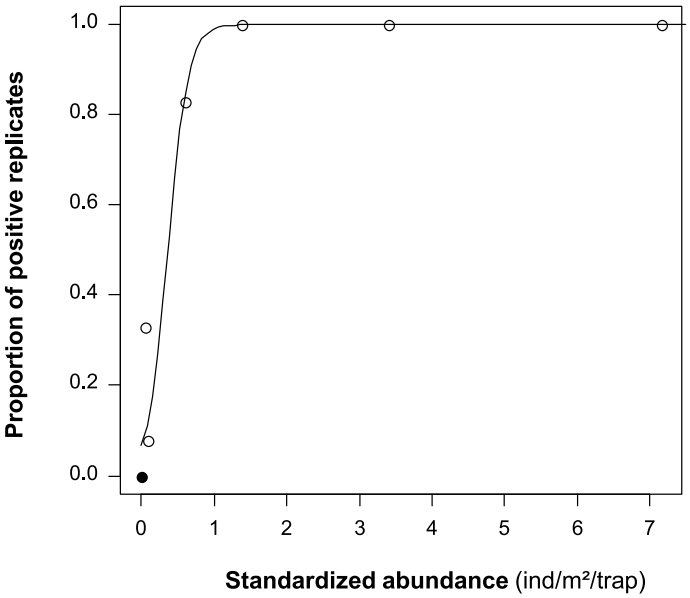

Figure 1. Relationships between the standardized abundance of adult *Xenopus laevis* caught in funnel traps over 3 consecutive days, and the number of positive replicates, i.e. the number of times the species DNA has been amplified in water samples. The line represents predictions from a generalized linear model.

side the colonized range, and the extraction and PCR controls were negative. Presence was detected in all 6 ponds from the known colonized range. Mean detection rate was 0.83, ranging from 0.08 (1/12 PCR replicates) to 1.0 (12/12 PCR replicates). We caught between 2 and 358 adults in the colonized ponds. *X. laevis* was detected using the eDNA technique in ponds where standardized abundance was as low as 0.085 individual.trap/m^2 (range 0.085-7.160) respectively. We found a strong positive relationship between the proportion of positive PCR replicates and standardized abundance (Binomial GLM: Deviance = 105.74, $P < 0.001$, fig. 1).

We successfully used eDNA to detect the presence of *X. laevis* in natural water bodies within their invasive range. Detection was confirmed in ponds where density was as low as 1 individual per 100 m^2. Owing to the sensitivity of the molecular method, it is thus possible to ascertain presence at the early stage of colonisation of a pond, a desirable characteristic in suspected cases of introduction. Thus, eDNA technique appears as a novel method able to efficiently survey the colonization process of new areas by *X. laevis*. It remains to be tested whether the method also works in lotic environments where the species can breed as in Portugal, but it has been usccessfully used for other stream amphibians (Pilliod et al., 2013).

We also found a positive relationship between the estimated density of adults and the rate of positive PCR amplifications. Similarly, eDNA concentration was used to estimate the biomass of a fish in freshwater lagoons (Takahara et al., 2012), and was significantly correlated with census size of two amphibian species in ponds (Thomsen et al., 2012). Thus, it may be feasible to derive a density index to record variation in census size to estimate the efficiency of control actions for instance. Additional tests are required to assess the sensitivity of the method to environmental variations (dilution, chemical composition of water, substrate type) before deriving a quantitative estimator of abundance though. Owing to analytical costs (circa 150 €/sample), eDNA survey may appear more expensive than classical surveys. However, the difference fades as travel costs and time spent

increase. A single site visit is required for the former technique and usually several for the latter, especially in secretive species or under unstable climates. In Western Europe, activity and therefore trapping efficiency of *X. laevis* is low about half of the year (Measey, 1998). Rain, cold spells, and wind also limit detection probability in acoustic surveys of anurans. In contrast, the persistence of DNA in water reduces time constraint and dependency to climatic conditions. Fieldwork can thus be planned ahead with limited uncertainty about its completion.

Formerly, eDNA methods have been used to survey species of conservation interest (Thomsen et al., 2012). This study and others outline their usefulness to survey invasive species (Dejean, 2012). The sensitivity, reliability, and ease of sampling of these novel techniques are crucial for the early detection of alien amphibians such as *X. laevis*. They may also give insight about the dynamics and dispersion of populations, which information is currently difficult to obtain for *X. laevis*, and more largely about the ecology of invasive amphibians on their introduced ranges (Darling and Mahon, 2011; Yoccoz, 2012).

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