Pôle départemental de Recherche sur la biodiversité en Isère

Appel à proposition 2011

Détection de l'Ecrevisse à pattes blanches (*Austropotamobius pallipes*) grâce à l'ADN environnemental

- Bilan de l'étude complémentaire 2012 -

UMR 5553
LABORATOIRE D'ECOLOGIE ALPINE
Université de Savoie,
UFR CISM, 73 376 La Bourget du Lac

Contexte du projet :

L'évaluation de la répartition des espèces est une première phase critique des études sur la biodiversité et est nécessaire pour de nombreuses disciplines telles que la biogéographie, la biologie de la conservation et l'écologie. Cependant, de nombreuses espèces sont difficiles à détecter, à des moments ou à des phases de développements particuliers.

Nous développons au laboratoire une nouvelle approche, fondée sur la persistance de l'ADN dans l'environnement, pour détecter la présence d'une espèce dans l'eau douce. En utilisant des amorces spécifiques amplifiant un fragment court de l'ADN mitochondrial, nous avons détecté la présence d'une grenouille (*Rana catesbeiana*) dans des environnements contrôlés (aquariums) et dans le milieu naturel. Cette méthode, dite méthode de l'ADN environnemental, à permis de détecter l'ADN de cette espèce dans tous les environnements où elle était présente (mares et étangs), même à de faibles densités. La fiabilité des résultats a été démontrée par l'identification de fragments d'ADN amplifiés à l'aide de méthodes traditionnelles de séquençage et par des techniques de « pyrosequencing ». Comme l'environnement peut conserver l'empreinte moléculaire des espèces qui le peuplent, cette méthode devrait permettre la détection fiable d'organismes discrets dans les zones humides sans même faire d'observation directe.

Combinée avec le séquençage massif et le développement des techniques codes barres d'ADN permettant l'identification de très nombreuses espèces, cette méthode particulièrement novatrice ouvre des perspectives prometteuse pour l'évaluation de la biodiversité actuelle.

Nous travaillons actuellement à la quantification de la rémanence de l'ADN dans le milieu aquatique. Les expériences du laboratoire d'Ecologie Alpine montrent qu'au laboratoire et en milieu semin-naturel l'ADN d'une espèce aquatique (têtard de Grenouille taureau et esturgeon) devient indétectable après environ deux semaines. Cette méthode permet donc de détecter les espèces « en place » dans les milieux. Elle est actuellement utilisée pour vérifier l'efficacité d'un programme d'éradication de la Grenouille taureau dans le Sud-Ouest de la France, et l'inventaire de nombreuses espèces aquatiques et semi-aquatiques.

Notre proposition à l'appel d'offre 2011 du Pôle départemental de Recherche sur la biodiversité en Isère était l'étude de faisabilité de cette méthode dans un milieu courant

(ruisseau) et sur une espèce patrimoniale (Ecrevisse à pied blanc). C'est donc une proposition d'ordre méthodologique, portée par un laboratoire de recherche et les fédérations de pêche des départements de l'Isère, de la Savoie et de la Haute-Savoie. Ces fédérations sont actuellement impliquées dans un programme régional sur l'écrevisse à pied blanc, et on comprendra l'enjeu important que représente l'amélioration des méthodes d'inventaire pour la connaissance de la distribution de cette espèce dans la région, les mesures de gestion et leurs évaluations qui pourraient en découler.

Méthodologie :

1) Définition d'une amorce spécifique pour l'écrevisse à patte blanche

De par son statut d'espèce patrimoniale, rare ou en danger dans de nombreux pays d'Europe de l'Ouest, cette espèce fait l'objet de nombreuses études démographiques et génétiques, plans de restauration, etc. Le laboratoire Ecologie, Evolution, Symbiose (UMR CNRS 6556) de l'Université de Poitiers étudie ainsi la génétique des populations et la phylogéographie de cette espèce (références ci-dessous). Il est maintenant nécessaire de vérifier quelle portion de l'ADN de l'écrevisse à pied blanc est bien spécifique de cette espèce puisque les analyses vont se faire à partir d'échantillons d'eau qui contiennent des fragments d'ADN de nombreuses autres espèces. Ce travail a été réalisé par une approche bioinformatique en collaboration avec Eric Coissac (MCF au LECA de Grenoble). A partir d'un tissu extrait sur une Ecrevisse à pattes blanches (banque de données d'échantillons du LECA), l'ADN mitochondrial a été extrait et amplifié. Une séquence courte de cet ADN a été identifiée grâce au programme développé au LECA et sa spécificité a été testé par PCR virtuelle dans les banques de données génétiques.

2) Persistance de l'ADN dans le milieu aquatique

Deux espèces ont été utilisées pour évaluer la persistance de l'ADN dans l'eau : des têtards de Grenouille taureau ont été étudiés afin de valider la possibilité d'intégrer l'approche de Ficetola et al. (2008) dans une stratégie d'inventaire en milieu stagnant. En raison du risque d'invasion et / ou transmission d'agents pathogènes pour les populations indigènes (par exemple *Batrachochytrium dendrobatidis* et *Ranavirus*), les têtards de Grenouille taureau ne

peuvent pas être utilisés en extérieur. Les expériences ont donc été réalisées dans des aquariums (comme dans Ficetola et al. (2008)). L'esturgeon de Sibérie a été utilisé comme espèce source d'ADN pour tester la persistance de l'ADN dans des conditions de terrain. Ils ont été placés dans des étangs artificiels créés il y a près de 20 ans sur le campus de l'Université de Savoie (où cette espèce n'a jamais été présente).

Pour l'expérience avec les têtards de Grenouille taureau, 3 densités de têtards ont été utilisés : 1, 5 et 10 têtards élevés dans des cristallisoirs de 900 mL pendant 5 jours, chaque densité étant répliquée 5 fois. Un cristallisoir de 900 ml sans têtards a été utilisé comme contrôle. Au cinquième jour, les têtards ont été enlevés. Toutes les 24 heures et pendant 20 jours, 15 ml d'eau ont été prélevés dans chaque cristallisoir. La température ambiante est maintenue constante tout au long de la durée de l'expérience (soit 17° ± 1°C).

Pour l'expérience avec les esturgeons, trois étangs de dimensions 12 m² et 0,40 m de profondeur ont été utilisés. Dans chaque bassin, un esturgeon (20 cm de long) a été placé pendant 10 jours. Le dixième jour, les esturgeons ont été enlevés et 15 ml d'eau ont été échantillonnés dans chaque étang. Des échantillons d'eau ont été prélevés toutes les 24 heures pendant 14 jours. La température de l'eau a fluctué entre 8 et 11°C durant cette période.

La durée de chaque expérience a été déterminée après un essai préliminaire suivant le même protocole mais sans réplication.

Pour les deux expériences, une solution composée de 1,5 ml d'acétate de sodium 3 M et 33 ml d'éthanol absolu est ajouté immédiatement après le prélèvement d'eau, puis stockée à – 20°C jusqu'à l'extraction de l'ADN.

3) Tests de différentes méthodes d'échantillonnage sur des cours d'eau

L'expérimentation consiste à placer une espèce source (esturgeon) dans un cours d'eau et de réaliser des prélèvements d'eau en aval (fig. 1)

La stratégie d'échantillonnage mise en œuvre pour les premières expérimentations consistait à prélever 15 ml d'eau toutes les 2 minutes à 2 mètres en val du poisson en cage.

Une autre stratégie de prélèvement a été testée : différents volumes d'eau ont été prélevés puis filtrer afin de recueillir les fragments d'ADN, et permettant de travailler sur des volumes beaucoup plus importants (50 à 100 litres) : l'eau est prélevée à l'aide d'une pompe péristaltique de terrain, et passe dans une capsule contenant un filtre dont les pores ont une

taille 0,45 µm. Ce filtre permet de retenir les cellules et les mitochondries libérées dans le milieu, et par là même l'ADN qu'elles contiennent. Des premiers tests nous ont permis de vérifier la faisabilité de ce type de prélèvement d'eau sur le terrain.

Figure 1 – Nasse contenant un Esturgeon pour l'expérience de détection de l'ADN en milieu courant.

4) Test de la détection de l'écrevisse à pattes blanches sur des ruisseaux où la présence de l'espèce est avérée.

Nous proposons de tester la méthode de l'ADNe dans les ruisseaux des trois ENS d'isère suivants :

- Le Marais de la Léze (Chantesse). Il s'agit d'un ENS associatif (géré par Avenir). La présence de *A. pallipes* a été confirmée en 2011 (Jean-Luc GROSSI et Mathieu JUTON).
- Le Marais des Sagnes (Le Sappey en Chartreuse). Il s'agit d'un ENS communal. La connaissance sur la présence de *A. pallipes* repose sur des données bibliographiques et des communications orales d'habitants et élus de la commune.

- Chirens / Val d'Ainan. C'est un ENS communal. La présence de *A. pallipes* n'est pas connue, mais les ruisseaux présentent des habitats favorables.

Résultats :

1) Définition d'une amorce spécifique pour l'écrevisse à patte blanche

A partir d'un tissu extrait sur une Ecrevisse à pattes blanches (banque de données d'échantillons du LECA), l'ADN mitochondrial a été extrait et amplifié avec succès. Une séquence courte de cet ADN a été identifiée grâce au programme développé au LECA et sa spécificité par ensuite été testé par PCR virtuelle dans les banques de données génétiques (e.g. GenBank).

2) Persistance de l'ADN dans le milieu aquatique

Expérience avec les têtards de Grenouille taureau : l'ADN a été détecté après que les têtards aient été enlevés, aux trois densités. La détectabilité de l'ADN dépend du temps écoulé depuis la suppression de la présence de la source, et de la densité de têtards « source » au début de l'expérience. La détectabilité de l'ADN ($z = 28.032$ et p 0,001) est corrélée négativement avec le temps. La densité de têtards, bien que significative, n'affiche aucune tendance (pas de différence entre une et cinq têtards, $z = 21,916$, $p = 0,0553$, alors que la détectabilité de l'ADN était plus élevé avec 10 têtards contre 1 têtard, $z = 2,091$, $p = 0,0365$). Après la suppression des têtards, l'ADN a été détecté jusqu'à 25 jours, avec une détectabilité supérieure à 5% (toutes les densités de têtards ensemble; Figure 2a).

Expérience avec les esturgeons : la détectabilité de l'ADN diminue avec le temps ($z = 26,136$ et p, 0,001, $R^2 = 0,5$). L'ADN est détecté jusqu'à 14 jours, avec une détectabilité supérieure à 5% (Figure 2b). Après 17 jours, ne pas détecter les fragments d'ADN courts de l'esturgeon présente une probabilité supérieure à 95%, et de 99% après 21 jours.

Ces résultats ont fait l'objet d'une publication : DEJEAN T et al. 2011 - PLoS ONE 6(8): e23398. doi:10.1371/journal.pone.0023398.

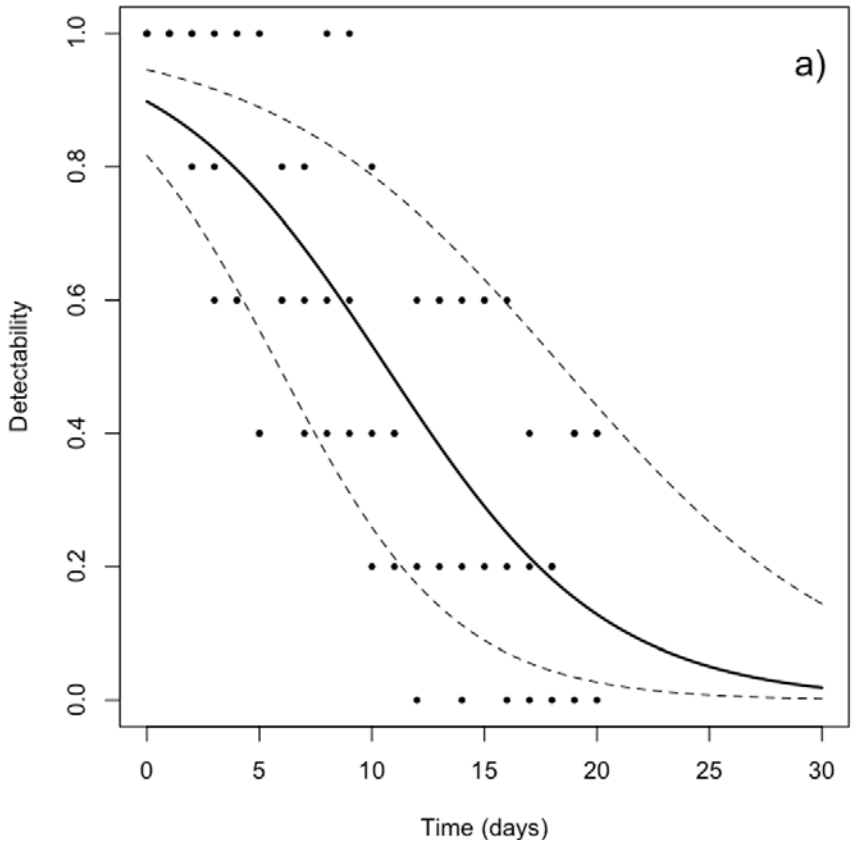

Figure 2a – Evolution de la détection de l'ADN de têtards de Grenouille taureau avec le temps. Les têtards sont enlevés du milieu (cristallisoirs) où l'eau est prélevée au temps t0.

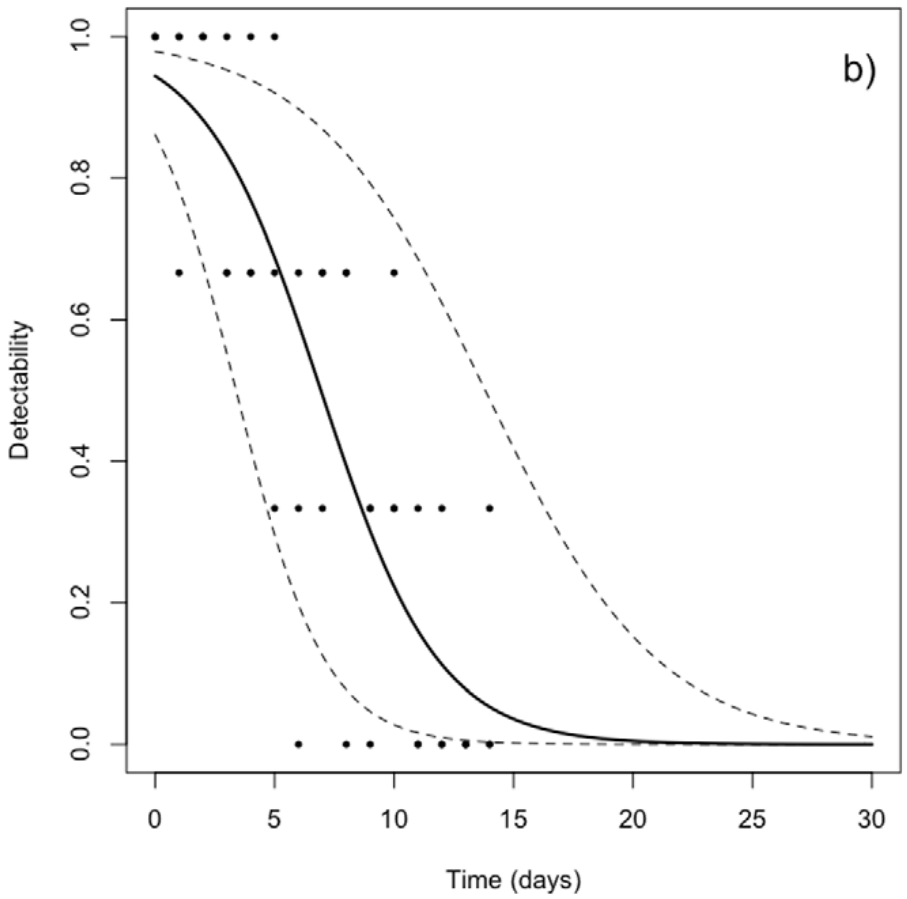

Figure 2b – Evolution de la détection de l'ADN des Esturgeons avec le temps. Les poissons sont enlevés du milieu (étangs) où l'eau est prélevée au temps t0.

3) Tests de différentes méthodes d'échantillonnage sur des cours d'eau

La méthode de détection de l'ADN environnemental a été validée pour un vertébré aquatique, la Grenouille taureau, en milieu stagnant (Ficetola et al., 2008). Notre objectif est de la mettre au point dans un milieu courant (ruisseau et rivière) et sur une espèce comme l'écrevisse à pied blanc pouvant présenter des densités faibles.

Les résultats de cette expérience permettent d'orienter le protocole d'échantillonnage en milieu courant : l'ADN d'une espèce cible à très faible densité (un seul individu dans une cage) est détecté d'une manière relativement aléatoire avec des prélèvements ponctuels (Figure 3). Ces résultats nous ont permis de mettre au point une nouvelle stratégie basée sur un prélèvement intégrateur réalisé à l'aide d'une pompe péristaltique (Figure 4).

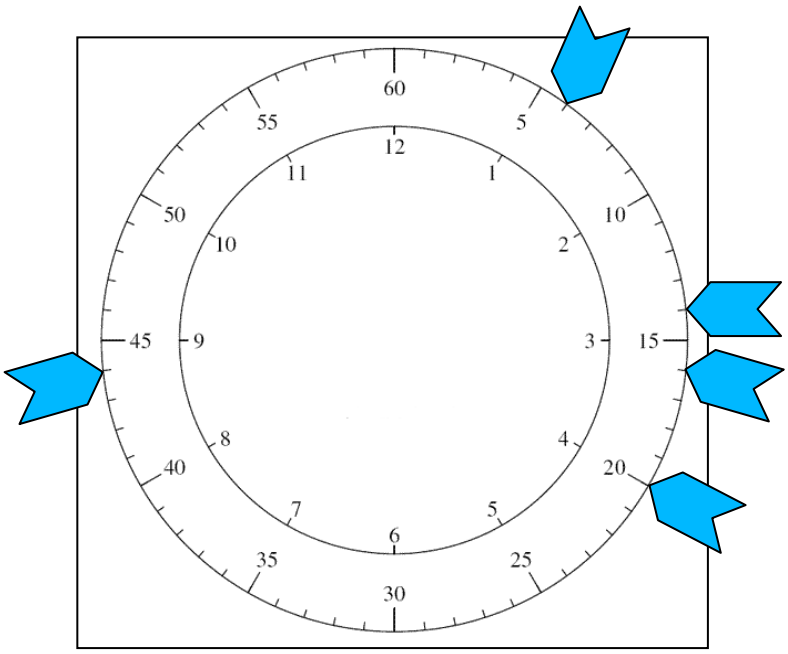

Figure. 3 - Un prélèvement de 15 ml d'eau est réalisé toutes les 2 min à 2 mètres en aval de la source ADN. Une PCR est réalisée par prélèvement. On observe des PCR+ pour l'esturgeon à 6, 14, 16, 20 et 44 min.

Figure 4. Dispositif de prélèvement intégrateur portable testé dans le cadre de cette étude.

Pour l'écrevisse à pied blanc, nous avons réalisé une expérience test pour détecter la séquence d'Ecrevisse dans un petit volume (aquarium) ayant contenu une Ecrevisse, suivant

un protocole mis au point précédemment pour tester la détection de l'ADN dans l'eau (Dejean et al., PlosOne, 2011, voir ci-dessus)).

Nous avons réalisé une campagne de prélèvement d'eau dans un ruisseau où la présence d'une population d'Ecrevisse est attestée (données de la fédération de pêche du département de la Savoie). Le protocole d'échantillonnage appliquée est celui décrit dans Ficetola et al., 2008, à savoir des prélèvements de 15 ml d'eau sur lesquels 3 PCR successives sont réalisées.

Les résultats obtenus sont très variables : la répétabilité n'est pas acquise entre les PCR appliquées sur le même échantillon d'eau. La méthode d'échantillonnage utilisée ne permet pas de décrire une méthode fiable et reproductible.

Nous proposons de mettre en place à l'automne 2013 une campagne d'échantillonnage (avec la méthode intégrative et d'autres méthodes de prélèvements d'eau développés dans d'autres programmes en cours) sur les cours d'eau accueillant des populations d'Ecrevisses de densité variable (voir ci-dessous).

4) Test de la détection de l'écrevisse à pattes blanches sur des ruisseaux où la présence de l'espèce est avérée.

Les résultats obtenus dans le cadre de ce projet financé par le CG 38 (détermination de l'amorce spécifique Ecrevisse à pieds blancs, test de la persistance de l'ADN dans l'eau, test de méthode de prélèvements en milieu courant, permettent maintenant de passer à la phase de test sur le terrain.

Cette étude est programmée pour l'automne 2013 sur trois Espaces Naturels Sensibles de l'Isère. Les résultats seront communiqués sous la forme d'un ajout à ce rapport.

Références bibliographiques :

DEJEAN T., A. VALENTINI, C. MIQUEL, P. TABERLET, E. BELLEMAIN & C. MIAUD, 2012 - Improved detection of an alien invasive species through environmental DNA barcoding: the example of the American bullfrog Lithobates catesbeianus. **Journal of Applied Ecology** 49(4): 953-959

DEJEAN T., VALENTINI A., DUPARC A., PELLIER-CUIT S., POMPANON F., TABERLET P. & MIAUD C., 2011 - Persistence of Environmental DNA in Freshwater Ecosystems. **PLoS ONE** 6(8): e23398. doi:10.1371/journal.pone.0023398.

FICETOLA G.F., MIAUD C., POMPANON F. & P. TABERLET, 2008 - Species detection using environmental DNA from water samples. **Biology Letters** 4: 423-425.

GRANDJEAN F. & SOUTY-GROSSET C. (2000) *Mitochondrial DNA variation and population genetic structure of the white-clawed crayfish, Austropotamobius pallipes*. **Conserv. Genet**. 1, 309-319.

GOUIN N., GRANDJEAN F. & SOUTY-GROSSET C. (2006) *Population genetic structure of the endangered crayfish Austropotamobius pallipes in France based on microsatellite variation : biogeographical inferences and conservation implications*. **Freshwater Biol.** 51, 1369-1387

MIAUD C., TABERLET P. & DEJEAN T., 2012 - ADN « environnemental » : un saut méthodologique pour les inventaires de la biodiversité**. Sciences Eaux & Territoires** 6: 92-95.

biology letters

Biol. Lett. (2008) **4**, 423–425
doi:10.1098/rsbl.2008.0118
Published online 9 April 2008

Population genetics

Species detection using environmental DNA from water samples

Gentile Francesco Ficetola[1,2,*], Claude Miaud[2], François Pompanon[1] and Pierre Taberlet[1]

[1]*Laboratoire d'Ecologie Alpine, CNRS-UMR 5553, Université Joseph Fourier, BP 53, 38041 Grenoble Cedex 09, France*
[2]*Laboratoire d'Ecologie Alpine, CNRS-UMR 5553, Université de Savoie, 73376 Le Bourget du Lac Cedex, France*
**Author and address for correspondence: Dipartimento di Scienze dell'Ambiente e del Territorio, Università Milano Bicocca, Piazza della Scienza 1, 20126 Milano, Italy (francesco.ficetola@unimi.it).*

The assessment of species distribution is a first critical phase of biodiversity studies and is necessary to many disciplines such as biogeography, conservation biology and ecology. However, several species are difficult to detect, especially during particular time periods or developmental stages, potentially biasing study outcomes. Here we present a novel approach, based on the limited persistence of DNA in the environment, to detect the presence of a species in fresh water. We used specific primers that amplify short mitochondrial DNA sequences to track the presence of a frog (*Rana catesbeiana*) in controlled environments and natural wetlands. A multi-sampling approach allowed for species detection in all environments where it was present, even at low densities. The reliability of the results was demonstrated by the identification of amplified DNA fragments, using traditional sequencing and parallel pyrosequencing techniques. As the environment can retain the molecular imprint of inhabiting species, our approach allows the reliable detection of secretive organisms in wetlands without direct observation. Combined with massive sequencing and the development of DNA barcodes that enable species identification, this approach opens new perspectives for the assessment of current biodiversity from environmental samples.

Keywords: biodiversity inventories; biological invasion; conservation genetics; DNA barcoding; secretive species

1. INTRODUCTION

The assessment of species distribution is a first critical phase of biodiversity studies and is necessary for several disciplines such as biogeography, conservation biology and ecology (Margurran 2004). However, several species are difficult to detect, especially during particular time periods or developmental stages, potentially biasing study outcomes (Gotelli & Colwell 2001; MacKenzie *et al.* 2006). The extraction of DNA from environmental samples allows the characterization of their micro-organisms (Venter *et al.* 2004). It can also provide information on extinct communities of macro-organisms, since short DNA sequences can persist for long time periods, as shown by the studies on old sediments, permafrost and ice cores (Hofreiter *et al.* 2003; Willerslev *et al.* 2003, 2007). While short DNA sequences may be present at high density in the environment, their potential for the study of present-day communities of macro-organisms remains substantially unexplored. Here we present a novel approach, based on the persistence of DNA in the environment, to detect the presence of a species in fresh water. We examined whether DNA fragments are preserved in the aquatic environment and whether they can be used for a reliable assessment of current species presence. We first used the method in controlled environments and then evaluated whether it could be applied under natural field conditions. The model species was the American bullfrog *Rana catesbeiana* (=*Lithobates catesbeianus*), an invasive amphibian for which high-quality census data exist (Ficetola *et al.* 2007*a*,*b*). This allowed reliable field validation. The American bullfrog is native to western North America, but has been introduced into ecosystems around the globe. It is considered one of the world's most harmful invasive species, since it is responsible for the decline of native amphibians by direct predation, competition, diffusion of diseases and complex biotic interactions (Blaustein & Kiesecker 2002; Kats & Ferrer 2003; Garner *et al.* 2006).

2. MATERIALS AND METHODS

We performed experiments in both controlled conditions and natural populations. Tadpoles were reared in aquariums filled with 3 l of water, collected in a natural alpine spring at 1000 m above sea level and at 80 km from the nearest bullfrog record. We used 0, 1, 5 and 10 tadpoles per aquarium; each density was replicated six times. After 24 hours, we collected a 15 ml water sample from each aquarium.

Bullfrog distribution in natural wetlands was assessed in France through traditional surveys of more than 2500 wetlands over 4 years (Ficetola *et al.* 2007*b*). We selected three ponds (surface 1000–10 000 m^2) where bullfrogs were present at low density (one to two adults seen, no reproduction), three ponds where bullfrogs were present at high density (dozens of adults and thousands of tadpoles) and three ponds where bullfrogs have never been detected. These latter ponds were more than 30 km from the nearest bullfrog record. For each pond, we collected three 15 ml water samples from different points.

Immediately after collection, we added 1.5 ml of sodium acetate 3 M and 33 ml absolute ethanol to the water samples; these were then stored at −20°C until DNA extraction. To recover the precipitated DNA and/or the cellular remains, we centrifuged the mixture (5500*g*, 35 min, 6°C) and discarded the supernatant (Valiere & Taberlet 2000). The pellet was then subjected to a classical DNA extraction using QIAamp Tissue Extraction Kit (Qiagen).

Polymerase chain reaction (PCR) amplification was performed with primers 5′-TGCCAACGGAGCATCATTC-3′ and 5′-ATAAA GGTAGGAGCCGTAGT-3′ especially designed for this experiment. These primers amplify a 79 bp segment of mitochondrial *cyt-b*, which is monomorphic in all 397 individuals analysed by population genetic studies covering the whole native and European range of the species (Ficetola *et al.* 2008). The basic local alignment search tool showed that these primers do not match with high scores to any other sequences stored in GenBank. Moreover, we used these primers to try amplifying tissue-extracted DNA of all other frog species living in France (*Rana ridibunda*, *Rana kleptonesculenta*, *Rana lessonae*, *Rana dalmatina*, *Rana temporaria*), with at least two individuals from different localities per each species. None of these tests resulted in DNA amplification. The PCRs were conducted in a 25 µl total volume with 10 mM Tris–HCl (pH 8.3), 50 mM KCl, 2.5 mM $MgCl_2$, 0.8 µM of each primer, BSA (5 µg), 0.5 U of AmpliTaq Gold DNA polymerase (Applied Biosystems) and 5 µl of DNA extract. The amplification of each water sample was repeated three to five times, using the multi-tube approach (Taberlet *et al.* 1996), which gave 9–15 repeats per pond. The number of replicates was similar in ponds with different bullfrog densities (mixed model, $F_{2,6}=0.506$, $p=0.51$). The PCR programme included an initial 10 min denaturation step at 95°C, 55 cycles of denaturation at 95°C for 30 s and annealing at

Electronic supplementary material is available at http://dx.doi.org/10.1098/rsbl.2008.0118 or via http://journals.royalsociety.org.

Received 3 March 2008
Accepted 25 March 2008

61°C for 30 s. PCR products were visualized using electrophoresis on 2% agarose gel. Sequencing reactions were performed in both directions using BigDye Terminator Cycle Sequencing Kit v. 1.1 and following standard procedures. Moreover, the PCR product of one pond was sequenced using the 454 pyrosequencing technology (GS20, Roche, Basel; Margulies *et al.* 2005). This massive parallel sequencing technique allows a large number of sequences to be obtained from a single PCR product, comparable with large-scale cloning protocols.

We used generalized mixed models assuming a binomial error to compare the amplification rates among ponds with different bullfrog densities. The identities of ponds and water samples were included as random factors, nested within density. Mixed models were fitted using lme4 in R (R Development Core Team 2006) and followed by *post hoc* orthogonal contrasts through the origin.

3. RESULTS

Using selective primers and PCR, we successfully amplified DNA from water samples of all 18 aquariums where we reared tadpoles at different densities (0.3, 1.7, 3.3 tadpoles per l), while we never amplified DNA from the aquariums where bullfrog tadpoles were absent. We also amplified DNA from water samples of all the natural ponds where the species was present, while we never amplified DNA from the ponds where bullfrogs were absent (table 1). Almost all PCR products were sequenced and corresponded perfectly to the published bullfrog *cyt-b* sequence (Austin *et al.* 2004). Moreover, 673 fragments from one PCR product were sequenced using the 454 pyrosequencing technology (Margulies *et al.* 2005). Apart from clearly recognizable sequencing and PCR errors (mostly in a poly A repeat), all obtained sequences perfectly matched the bullfrog *cyt-b*, indicating that the PCR products were not mixes of different sequences. The probability that all nine replicates from a pond with a low density of frogs would be negative (i.e. false negative) is approximately 1.5% (see below).

These results were obtained using a multi-tube approach and ancient DNA precautions, which are suitable for analysing DNA that is degraded and/or at low concentrations (Taberlet *et al.* 1996). In water samples collected in ponds where bullfrogs were present, 22–89% of replicates were positive (table 1). The average amplification success ($\pm$s.e.) was 0 in ponds where bullfrogs were absent, 0.37 ± 0.1 in ponds where bullfrogs were present at low densities and 0.79 ± 0.08 in ponds where bullfrogs were present at high densities. Owing to the protocol used (multi-tube approach and high number of PCR cycles), it is necessary to ensure that the positive amplifications were not due to artefacts. This requires that all controls be negative and that this lack of amplification not be due to chance. Differences in amplification rates among ponds with differing densities of target species were significant (generalized linear mixed model, $\chi^2_2 = 19.5$, $p < 0.0001$). The amplification success was significantly higher in ponds with bullfrogs than in control ponds (orthogonal contrast, $\chi^2_1 = 18.2$, $p < 0.0001$); it is thus unlikely that all the control samples were negative just by chance. Moreover, the amplification rate was significantly higher in ponds with high bullfrog densities than in ponds with low densities ($\chi^2_1 = 5.7$, $p = 0.017$).

Table 1. Rate of bullfrog detection in water samples.

pond	bullfrog presence and relative abundance	water samples positives at least once	positive PCRs
1	yes-low	2/3	2/9
2	yes-low	3/3	6/9
3	yes-low	2/3	2/9
4	yes-high	3/3	8/9
5	yes-high	3/3	6/9
6	yes-high	3/3	8/10
7	no	0/3	0/9
8	no	0/3	0/9
9	no	0/3	0/15

4. DISCUSSION

We showed that environmental DNA (either in solution or in cellular debris) can be used to ascertain species presence in a wetland, and that this technique is able to discriminate between absence and presence, even at low densities. As the environment can retain the molecular imprint of inhabiting species (Hofreiter *et al.* 2003), our approach allows the reliable detection of secretive organisms in wetlands without direct observation. This is possible because environmental DNA can be detected at very low concentrations (see electronic supplementary material). The same approach could be useful for studying secretive aquatic or semi-aquatic species, which release DNA into the environment through mucus, faeces, urine and remains.

This approach can be the answer to many situations where traditional census techniques give low-quality results and/or require a huge sampling effort. This is the case when trying to quantify secretive harmful, invasive (such as bullfrog) or threatened species. Detecting invasive species at the early stages of invasions, and when they are at low densities, is the key to timely interventions to control them (Hulme 2006). Moreover, this technique allows the assessment of distribution of rare threatened species that are the target of conservation plans, or of harmful species that are kept at low density by management, with a reduced monitoring effort.

The use of such a method, like all techniques dealing with the detection of DNA traces, requires several precautions (Taberlet *et al.* 1996; Cooper & Poinar 2000). First, several factors could affect the amount of DNA in environmental samples, such as volume of water, size and density of the organism and volume of secretions. Second, as it is difficult to evaluate how long DNA fragments persist in water, this method could lead to the detection of a species after it has left the wetland. Short DNA fragments can persist a long time under dry cold conditions and in the absence of light. In one extreme example, DNA from extinct vertebrates has been amplified from 10 000-year old dry cave sediments (Willerslev *et al.* 2003). Actually, DNA fragments of approximately 400 bp may persist up to one week at 18°C in lake water (Matsui *et al.* 2001). This suggests that detecting a species that is no longer present is unlikely, owing to both the DNA's fast decomposition rate and its degradation from UV radiation exposure.

However, the presence of cells or particle-bound DNA may lead to a longer persistence of detectable DNA in water.

In addition to its applications for the study and management of secretive freshwater species, this approach opens new avenues for the study of biodiversity. The ongoing effort to develop DNA barcodes for identifying species from degraded DNA (Hajibabaei *et al.* 2006; Taberlet *et al.* 2007) will make our approach applicable to more and more plant and animal species. Moreover, massive sequencing techniques could be used to analyse PCR products generated with universal primers working on degraded substrates (e.g. Hofreiter *et al.* 2003; Willerslev *et al.* 2003; Taberlet *et al.* 2007). These factors will soon make possible the assessment of the current biodiversity of macro-organisms from environmental samples.

G.F.F. was partially founded by a grant of the French Ministry for Research for young foreign researchers.

Austin, J. D., Lougheed, S. C. & Boag, P. T. 2004 Discordant temporal and geographic patterns in maternal lineages of eastern north American frogs, *Rana catesbeiana* (Ranidae) and *Pseudacris crucifer* (Hylidae). *Mol. Phylogenet. Evol.* **32**, 799–816. (doi:10.1016/j.ympev.2004.03.006)

Blaustein, A. R. & Kiesecker, J. M. 2002 Complexity in conservation: lessons from the global decline of amphibian populations. *Ecol. Lett.* **5**, 597–608. (doi:10.1046/j.1461-0248.2002.00352.x)

Cooper, A. & Poinar, H. N. 2000 Ancient DNA: do it right or not at all. *Science* **289**, 1139–1139. (doi:10.1126/science.289.5482.1139b)

Ficetola, G. F., Thuiller, W. & Miaud, C. 2007*a* Prediction and validation of the potential global distribution of a problematic alien invasive species—the American bullfrog. *Divers. Distrib.* **13**, 476–485. (doi:10.1111/j.1472-4642.2007.00377.x)

Ficetola, G. F., Coïc, C., Detaint, M., Berroneau, M., Lorvelec, O. & Miaud, C. 2007*b* Pattern of distribution of the American bullfrog *Rana catesbeiana* in Europe. *Biol. Invasions* **9**, 767–772. (doi:10.1007/s10530-006-9080-y)

Ficetola, G. F., Bonin, A. & Miaud, C. 2008 Population genetics reveals origin and number of founders in a biological invasion. *Mol. Ecol.* **17**, 773–782. (doi:10.1111/j.1365-294X.2007.03622.x)

Garner, T. W. J., Perkins, M. W., Govindarajulu, P., Seglie, D., Walker, S., Cunningham, A. A. & Fisher, M. C. 2006 The emerging amphibian pathogen *Batrachochytrium dendrobatidis* globally infects introduced populations of the North American bullfrog, *Rana catesbeiana*. *Biol. Lett.* **2**, 455–459. (doi:10.1098/rsbl.2006.0494)

Gotelli, N. J. & Colwell, R. K. 2001 Quantifying biodiversity: procedures and pitfalls in the measurement and comparison of species richness. *Ecol. Lett.* **4**, 379–391. (doi:10.1046/j.1461-0248.2001.00230.x)

Hajibabaei, M., Smith, M. A., Janzen, D. H., Rodriguez, J. J., Whitfield, J. B. & Hebert, P. D. N. 2006 A minimalist barcode can identify a specimen whose DNA is degraded. *Mol. Ecol. Notes* **6**, 959–964. (doi:10.1111/j.1471-8286.2006.01470.x)

Hofreiter, M., Mead, J. I., Martin, P. & Poinar, H. N. 2003 Molecular caving. *Curr. Biol.* **13**, R693–R695. (doi:10.1016/j.cub.2003.08.039)

Hulme, P. E. 2006 Beyond control: wider implications for the management of biological invasions. *J. Appl. Ecol.* **43**, 835–847. (doi:10.1111/j.1365-2664.2006.01227.x)

Kats, L. B. & Ferrer, R. P. 2003 Alien predators and amphibian declines: review of two decades of science and the transition to conservation. *Divers. Distrib.* **9**, 99–110. (doi:10.1046/j.1472-4642.2003.00013.x)

MacKenzie, D. I., Nichols, J. D., Royle, J. A., Pollock, K. H., Bailey, L. A. & Hines, J. E. 2006 *Occupancy estimation and modeling: inferring patterns and dynamics of species occurrence*. Amsterdam, The Netherlands: Elsevier.

Margulies, M. *et al.* 2005 Genome sequencing in microfabricated high-density picolitre reactors. *Nature* **437**, 376–380. (doi:10.1038/nature03959)

Margurran, A. E. 2004 *Measuring biological diversity*. Malden, MA: Blackwell Science.

Matsui, M., Honjo, M. & Kawabata, Z. 2001 Estimation of the fate of dissolved DNA in thermally stratified lake water from the stability of exogenous plasmid DNA. *Aquat. Microb. Ecol.* **26**, 95–102. (doi:10.3354/ame026095)

R Development Core Team 2006 *R: a language and environment for statistical computing*. Vienna, Austria: R Foundation for Statistical Computing.

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

Taberlet, P. *et al.* 2007 Power and limitations of the chloroplast trnL (UAA) intron for plant DNA barcoding. *Nucleic Acids Res.* **35**, e14. (doi:10.1093/nar/gkl938)

Valiere, N. & Taberlet, P. 2000 Urine collected in the field as a source of DNA for species and individual identification. *Mol. Ecol.* **9**, 2150–2152. (doi:10.1046/j.1365-294X.2000.11142.x)

Venter, J. C. *et al.* 2004 Environmental genome shotgun sequencing of the Sargasso sea. *Science* **304**, 66–74. (doi:10.1126/science.1093857)

Willerslev, E. *et al.* 2003 Diverse plant and animal genetic records from Holocene and Pleistocene sediments. *Science* **300**, 791–795. (doi:10.1126/science.1084114)

Willerslev, E. *et al.* 2007 Ancient biomolecules from deep ice cores reveal a forested Southern Greenland. *Science* **317**, 111–114. (doi:10.1126/science.1141758)

OPEN ACCESS Freely available online

PLoS one

Persistence of Environmental DNA in Freshwater Ecosystems

Tony Dejean[1,2,3], Alice Valentini[1,2], Antoine Duparc[2], Stéphanie Pellier-Cuit[4], François Pompanon[4], Pierre Taberlet[4], Claude Miaud[2]*

1 SPYGEN, Savoie Technolac - BP 274, Le Bourget-du-Lac, France, **2** Laboratoire d'Ecologie Alpine, UMR CNRS 5553, Université de Savoie, Le Bourget-du-Lac, France, **3** Parc Naturel Régional Périgord-Limousin, La Coquille, France, **4** Laboratoire d'Ecologie Alpine, UMR CNRS 5553, Université Grenoble I, Grenoble, France

Abstract

The precise knowledge of species distribution is a key step in conservation biology. However, species detection can be extremely difficult in many environments, specific life stages and in populations at very low density. The aim of this study was to improve the knowledge on DNA persistence in water in order to confirm the presence of the focus species in freshwater ecosystems. Aquatic vertebrates (fish: Siberian sturgeon and amphibian: Bullfrog tadpoles) were used as target species. In control conditions (tanks) and in the field (ponds), the DNA detectability decreases with time after the removal of the species source of DNA. DNA was detectable for less than one month in both conditions. The density of individuals also influences the dynamics of DNA detectability in water samples. The dynamics of detectability reflects the persistence of DNA fragments in freshwater ecosystems. The short time persistence of detectable amounts of DNA opens perspectives in conservation biology, by allowing access to the presence or absence of species e.g. rare, secretive, potentially invasive, or at low density. This knowledge of DNA persistence will greatly influence planning of biodiversity inventories and biosecurity surveys.

Citation: Dejean T, Valentini A, Duparc A, Pellier-Cuit S, Pompanon F, et al. (2011) Persistence of Environmental DNA in Freshwater Ecosystems. PLoS ONE 6(8): e23398. doi:10.1371/journal.pone.0023398

Editor: Jack Anthony Gilbert, Argonne National Laboratory, United States of America

Received May 25, 2011; **Accepted** July 15, 2011; **Published** August 8, 2011

Funding: Tony Dejean was given a grant by the Ministry of Research (Grant CIFRE Number 966/2007), and Claude Miaud by the European Community (Marie Curie OIF grant Number 1018). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have read the journal's policy and have the following conflicts: Two authors (Tony Dejean and Alive Valentini) are working for a private company (SPYGEN) but this does not alter the adherence of all authors to all the PLoS ONE policies on sharing data and materials.

* E-mail: claude.miaud@univ-savoie.fr

Introduction

The precise knowledge of species distribution is a key point for conservation strategies, especially when the focal species are invasive, threatened or endangered [1–4]. However, its detection may be extremely difficult in many environments, at specific life stages and in populations at very low densities [5,6]. To overcome this problem, DNA barcoding was recently used in order to detect species through extracellular DNA present in environmental samples, coming from cell lysis or living organism excretion or secretion [7]. This method allows species presence detection, without any contact (e.g. visual, auditory) when the only available indicators are hair, faeces or urine left behind by the organisms. For example, faeces and hair samples were used for monitoring the recent wolf range expansion in France and Switzerland [8]. In aquatic ecosystems, Ficetola et al. [5] proposed a new methodology for species detection using environmental DNA from freshwater samples. The aim was to detect the American bullfrog (*Rana catesbeiana* = *Lithobates catesbeianus*) in natural ponds in SW France where it was introduced about 40 years ago [9]. The method, efficient in detecting frogs even at very low density, can be integrated into the eradication strategy of this invasive species to estimate its distribution in ponds before and after frog removal. Environmental DNA could thus be used for a biodiversity inventory (e.g. introduced Asian carp in North America [10])but also to control the efficiency of eradication actions. In this context, the precise assessment of the species presence requires knowledge of DNA persistence in water.

DNA persistence can be defined as the continuance of DNA after the removal of its source. However, any detection in the field is always imperfect and sampling is a stochastic process [11]. Detection probability depends on the species density and on the ratio between the DNA released by the organism and the DNA degraded by environmental factors.

In this study we estimated the time of DNA detection taking into account aquatic environment conditions and DNA concentrations. Experimentation was performed on two different species: the American bullfrog (*Rana catesbeiana* = *Lithobates catesbeianus*) and the Siberian sturgeon (*Acipenser baerii*).

Materials and Methods

Tests conditions and sampling

Two species were used for assessing the persistence of detectable amounts of DNA. Bullfrog tadpoles were studied to validate the possibility of integrating the approach of Ficetola et al. [5] in the species eradication strategy. Due to the risk of invasion and/or pathogen transmission to native populations (e.g. *Batrachochytrium dendrobatidis* [12] and Ranavirus [13]) bullfrog tadpoles cannot be used outdoors, and the experiments were performed in aquariums (as in Ficetola et al. [5]). The siberian sturgeon was used as DNA source species to test field conditions and placed in artificial ponds

created about 20 years ago on the University campus, where this species has never been present.

For the bullfrog experiment, 3 different densities of tadpoles were used. One, 5 and 10 tadpoles were reared in 900 mL glass beakers for 5 days and each density was replicated 5 times. A 900 mL glass beaker without tadpoles was used as control. At the fifth day, the tadpoles were removed. At this time and every 24 h during 20 days, 15 mL of water were sampled from each glass beaker. Room temperature was maintained constant throughout the experimental period and the water temperature measured in the glass beakers was $17\pm1^{\circ}C$.

For the sturgeon experiment, three ponds of dimensions 12 m^2 and 0.40 m deep were used. In each pond, a sturgeon (20 cm long) was housed for 10 days (from November the 04^{th} to 13^{th} 2009). On the tenth day, the sturgeons were removed and 15 mL of water were sampled from each pond. Water samples were collected every 24 h during 14 days. Water temperature fluctuated from 8 to $11^{\circ}C$ during this period.

The duration of each experiment was determined after a preliminary test on the same condition without replication. In both experiments, water samples were added to a solution composed of 1.5 mL of sodium acetate 3 M, and 33 mL absolute ethanol immediately after collection, and then stored at $-20^{\circ}C$ until the DNA extraction.

DNA analysis

DNA extraction was adapted from Ficetola et al. [5]: we centrifuged the mixture at 9400 g for 1 h at $6^{\circ}C$ to recover DNA and/or the cellular remains. The DNA from the pellet was extracted using QIAmp Blood and Tissue Extraction Kit (Qiagen, GmbH, Hilden, Germany), following manufactures' instructions. DNA extraction was performed in a dedicated room for degraded DNA samples. Control extractions were systematically performed to monitor possible contaminations.

Bullfrog DNA was amplified with primers described in Ficetola et al. [5]. Sturgeon DNA was amplified with primers designed to amplify a 98 bp fragment of the *Acipenser mt*DNA control region (5′ – GACAGTAATTGTAGAGTTTC - 3′and 5′ – CAGTAACAG-GCTGATTATG - 3′). *In silico* PCR, performed using the ecoPCR software [14] (http://www.grenoble.prabi.fr/trac/ecoPCR) on the whole GenBank dataset extracted on July 9 2009, showed the suitability of the primer pair. The only 4 species amplified were from the genus *Acipenser*: *A. persicus*, *A. brevirostrum*, *A. gueldenstaedtii* and *A. baerii*, the latter was the only species present in the ponds.

DNA amplifications were carried out in a final volume of 25 µL, using 3 µL of DNA extract as template. The amplification mixture contained 1 U of Ampli*Taq* Gold DNA Polymerase (Applied Biosystems, Foster City, CA), 10 mM Tris-HCl, 50 mm KCl, 2 mM of MgCl2, 0.2 mM of each dNTPs, 0.2 µm of each primer, and 0.005 mg of bovine serum albumin (BSA, Roche Diagnostics, Basel, Switzerland). After 10 min at $95^{\circ}C$ (*Taq* activation), the PCR cycles were performed as follows: 55 cycles of 30 s at $95^{\circ}C$, 30 s at $54^{\circ}C$ for *A. baerii* and $61^{\circ}C$ for *R. catesbeiana* primer pair. The amplification for the sturgeon experiments was repeated 3 times using multi-tube approach [15]. PCR products were visualized using electrophoresis on 2% agarose gel.

For the bullfrog experiment, the DNA detectability was defined as the number of positive samples detected among the 15 samples collected per day (5 replicates and 3 densities). For the Sturgeon experiment, the DNA detectability was defined as the number of positive samples detected among the 9 samples analysed (3 samples collected and 3 PCR per sample).

Statistical modelling

For the bullfrog experiment, the relationship between the DNA detectability, the time and density of tadpoles was inferred with a generalized linear model using binomial error. For the sturgeon experiment, the relationship between the DNA detectability and time was inferred with a linear mixed model with sites as random effect. In both experiments, a backward selection procedure was used starting with the full model containing all fixed explanatory components. Then, fixed variables were removed step by step. The best fitted model was selected based on AIC [16]. All analyses were done with R (R 2.10) [17].

Ethics Statement

The research presented has been approved by the Animal Care and Use Committee (permit #CMLECA5553 05/19/05) of the Savoie University at Le Bourget du Lac (France).

Results

In the bullfrog experiment, DNA was detected after tadpole removal at the three densities. DNA detectability was best explained by time and tadpole density factor. DNA detectability ($z=-8.032$ and $p<0.001$) was negatively correlated with time. Tadpole density, although significant, showed no trends according to levels of density (no difference between 1 and 5 tadpoles, $z=-1.916$, $p=0.0553$, while DNA detectability was higher with 10 tadpoles compared with 1 tadpole, $z=2.091$, $p=0.0365$). After the removal of tadpoles, the DNA was detected until day 25 with a detectability superior to 5% (all tadpole densities together; Figure 1a).

In the sturgeon experiment, DNA detectability was negatively correlated with time ($z=-6.136$ and $p<0.001$, $R^2=0.5$). DNA was detected until day 14 with a detectability superior to 5% (Figure 1b). Using 3 replicates per pond, after 17 days there is a probability higher than 95% to not detect short DNA fragments (i.e. the probability that all 3 replicates are negative) or 21 days if a 99% threshold is considered.

Discussion

Freshwater environments and oceans constitute a great reservoir of extracellular DNA [18]. Its detection in an aquatic environment depends on its release and degradation. The density of individuals influences the dynamics of DNA detectability in water samples, as shown by the bullfrog experiment in this study.

Once released from organisms, extracellular DNA in the environment may persist, adsorbed in organic or inorganic particles. It may also be transformed by competent soil microorganisms, or may be degraded (see [19] for a comprehensive review).

Several factors operate in DNA degradation. Endogenous nucleases, water, UV radiation and the action of bacteria and fungi in the environment contribute to DNA decay [20]. Different studies demonstrated that 300–400 bp fragments could be detected in water up to one week in controlled conditions [21–24]. Short DNA fragments are usually very slowly degraded and can be recovered from environmental samples [25]. They are well preserved in dry and cold environments and in the absence of light [20]. For example, the Greenland ancient communities of plants and animals was described using 450 000 year old silty ice samples extracted from the bottom of the Greenland ice cap [26]. In this study, using short fragments, DNA was detectable up to c. a. one month after the removal of its source, for both animal species used. This discrepancy in DNA persistence in for example soil and water and can be due to the action of endogenous nuclease and water

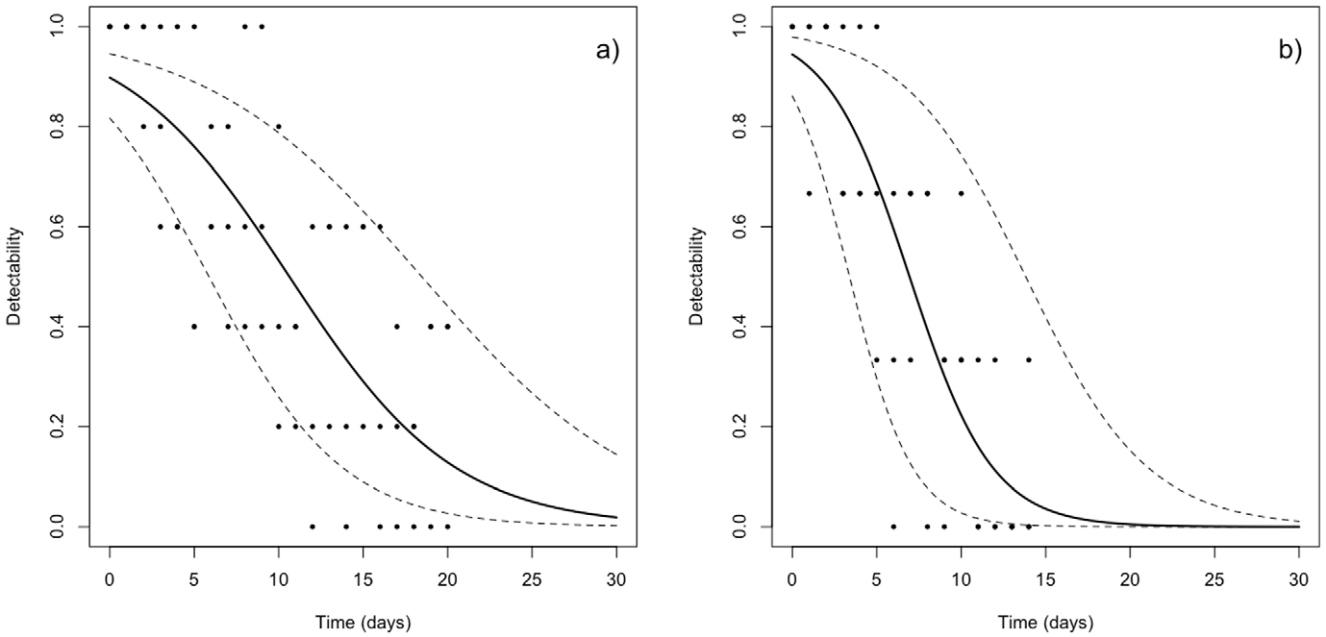

Figure 1. DNA detectability in water according to time. DNA detectability in water in control conditions (a) and in natural conditions (b) according to the time elapsed since the DNA source removal.
doi:10.1371/journal.pone.0023398.g001

that hydrolyses DNA molecules and creates DNA strand breaks by direct cleavage of the DNA phosphodiester backbone or breakage of the sugar backbone after depurination [27]. UV radiation [28] and DNA uptake by micro-organisms, as source of nutriments (carbon, nitrogen and phosphorous) and to repair their own DNA damages [29], contribute also to damage and decrease DNA molecules density. Microorganisms' uptake varies with temperature; and as a consequence, DNA detectability can vary according to the period of the year. In fact low temperature can slow down enzymatic and microbial activity resulting in slower DNA degradation [24].

DNA detection and, as a consequence, DNA persistence estimation, is influenced by sampling and analysis strategy, other than environmental factors. The sampling and the analysis strategy must be extremely rigorous. Before any environmental DNA analysis, the reliability and the robustness of primers must be tested. First, the analysis must be performed *in silico* (e. g. using ecoPCR software [14]) in order to insure primer specificity (e. g. other species were not amplified at the same time as the species of interest) [30]. Once specific primers were found, reliabilty must be tested on very high quality DNA (e.g. extracted from tissus samples), and PCR conditions must be otpimized. Environmental DNA is rare and preacautions similar to those used for ancient DNA analysis must be taken [31]. DNA must be extracted in a dedicated room for rare DNA, mock samples without DNA have to be analysed in parallel, as well as positive samples. PCR cycles have to be increased and high attention must to be taken to avoid contamination. The optimum strategy to enhance the reliability is to increase the number of analysed samples, i.e. more water samples in the field and more genetic replicates (multi-tube approach [15]) in the laboratory. However, all the sampling and analysis strategy must be adapted to the studied environments (e.g. large water bodies, marshes, etc) and species. In running waters, other sampling strategies will be developed, based e.g. on pumping water samples to increase DNA collection.

The dynamics of detectability reflects the persistence of DNA fragments in freshwater ecosystems. In this study we demonstrate that DNA persistence is less than one month. The short time persistence of detectable amounts of DNA opens new perspectives in conservation biology, by allowing access to the presence or absence of species e.g. rare, secretive, potentially invasive, or at low density. This knowledge of DNA persistence will greatly influence planning of biodiversity inventories and biosecurity surveys.

Acknowledgments

We thank Christian Miquel, Delphine Rioux, Carole Poillot and Nathalie Tissot for the support and suggestions during the study.

Author Contributions

Conceived and designed the experiments: TD CM. Performed the experiments: TD AV AD SP-C FP. Analyzed the data: TD AV AD FP. Lead writers of the paper: TD AV. Contributed to the writing of the paper: SP-C FP PT CM.

References

1. Harvey CT, Qureshi SA, MacIsaac HJ (2009) Detection of a colonizing, aquatic, non-indigenous species. Divers Distrib 15: 429–437.
2. Margurran AE (2004) Measuring Biological Diversity. Malden: Blackwell Science.
3. Mehta SV, Haight RG, Homans FR, Polasky S, Venette RC (2007) Optimal detection and control strategies for invasive species management. Ecol Econ 61: 237–245.
4. Smith DR (2006) Survey design for detecting rare freshwater mussels. J N Am Benthol Soc 25: 701–711.
5. Ficetola GF, Miaud C, Pompanon F, Taberlet P (2008) Species detection using environmental DNA from water samples. Biol Letters 4: 423–425.
6. MacKenzie DI, Nichols JD, Sutton N, Kawanishi K, Bailey LL (2005) Improving inferences in popoulation studies of rare species that are detected imperfectly. Ecology 86: 1101–1113.

7. Valentini A, Pompanon F, Taberlet P (2009) DNA barcoding for ecologists. Trends in Ecol Evol 24: 110–117.
8. Valiere N, Taberlet P (2000) Urine collected in the field as a source of DNA for species and individual identification. Mol Ecol 9: 2150–2152.
9. Détaint M, Coïc C (2001) Invasion de la Grenouille taureau (*Rana catesbeiana* Shaw) en France: Synthèse bibliographique - Suivi 2000–2001 - Perspectives. Le Haillan (33): Association Cistude Nature. 30 p.
10. Jerde C, Mahon A, Chadderton W, Lodge D (2011) Sight-unseen detection of rare aquatic species using environmental DNA. Conserv Lett 4: 150–157.
11. Nichols JD, Thomas L, Conn PB (2009) Inferences About Landbird Abundance from Count Data: Recent Advances and Future Directions. In: Patil GP, ed. Modeling Demographic Processes In Marked Populations. New York: Springer. pp 201–235.
12. Garner TWJ, Perkins MW, Govindarajulu P, Seglie D, Walker S, et al. (2006) The emerging amphibian pathogen Batrachochytrium dendrobatidis globally infects introduced populations of the North American bullfrog, Rana catesbeiana. Biol Letters 2: 455–459.
13. Une Y, Sakuma A, Matsueda H, Nakai K, Murakami M (2009) Ranavirus Outbreak in North American Bullfrogs (Rana catesbeiana), Japan, 2008. Emerg Infect Dis 15: 1146–1147.
14. Taberlet P, Coissac E, Pompanon F, Gielly L, Miquel C, et al. (2007) Power and limitations of the chloroplast trnL (UAA) intron for plant DNA barcoding. Nucl Acids Res 35: e14.
15. Taberlet P, Griffin S, Goossens B, Questiau S, Manceau V, et al. (1996) Reliable genotyping of samples with very low DNA quantities using PCR. Nucl Acids Res 24: 3189–3194.
16. Burnham KP, Anderson DR (2002) Model Selection and Multimodel Inference: A Practical Information-Theoretic Approach. New York: Springer.
17. Team RDC (2010) R: A Language and Environment for Statistical Computing. Vienna, Austria: R Foundation for Statistical Computing.
18. Potè J, Ackermann R, Wildi W (2009) Plant leaf mass loss and DNA release in freshwater sediments. Ecotox Environ Safe 72: 1378–1383.
19. Levy-Booth DJ, Campbell RG, Gulden RH, Hart MM, Powell JR, et al. (2007) Cycling of extracellular DNA in the soil environment. Soil Biol Biochem 39: 2977–2991.
20. Shapiro B (2008) Engineered polymerases amplify the potential of ancient DNA. Trends Biotechnol 26: 285–287.
21. Alvarez AJ, Yumet GM, Santiago CL, Toranzos GA (1996) Stability of manipulated plasmid DNA in aquatic environments. Environ Toxicol Water Qual 11: 129–135.
22. Matsui K, Honjo M, Kawabata Z (2001) Estimation of the fate of dissolved DNA in thermally stratified lake water from the stability of exogenous plasmid DNA. Aquat Microb Ecol 26: 95–102.
23. Romanowski G, Lorenz MG, Sayler G, Wackernagel W (1992) Persistence of free plasmid DNA in soil monitored by various methods, inclding a transformation assay. Appl Environ Microb 58: 3012–3019.
24. Zhu B (2006) Degradation of plasmid and plant DNA in water microcosms monitored by natural transformation and real-time polymerase chain reaction (PCR). Water Res 40: 3231–3238.
25. Deagle B, Eveson JP, Jarman S (2006) Quantification of damage in DNA recovered from highly degraded samples - a case study on DNA in faeces. Front Zool 3: 11.
26. Willerslev E, Cappellini E, Boomsma W, Nielsen R, Hebsgaard MB, et al. (2007) Ancient biomolecules from deep ice cores reveal a forested Southern Greenland. Science 317: 111–114.
27. Willerslev E, Cooper A (2005) Ancient DNA. P Roy Soc B-Biol Sci 272: 3–16.
28. Ravanat JL, Douki T, Cadet J (2001) Direct and indirect effects of UV radiation on DNA and its components. J Photoch Photobio B 63: 88–102.
29. Chen I, Dubnau D (2004) DNA uptake during bacterial transformation. Nat Rev Microbiol 2: 241–249.
30. Ficetola GF, Coissac E, Zundel S, Riaz T, Shehzad W, et al. (2011) An In silico approach for the evaluation of DNA barcodes. BMC Genomics 11: 434.
31. Cooper A, Poinar HN (2000) Ancient DNA: Do it right or not at all. Science 289: 1139–1139.

Journal of Applied Ecology

British Ecological Society

Journal of Applied Ecology doi: 10.1111/j.1365-2664.2012.02171.x

Improved detection of an alien invasive species through environmental DNA barcoding: the example of the American bullfrog *Lithobates catesbeianus*

Tony Dejean[1,2,3]*, Alice Valentini[1], Christian Miquel[4], Pierre Taberlet[4], Eva Bellemain[1] and Claude Miaud[2]†

[1]*SPYGEN, 12 allée du Lac de Garde, Bâtiment House Boat n°7, Savoie Technolac, 73370 Le Bourget du Lac, France;* [2]*Laboratoire d'Ecologie Alpine, UMR CNRS 5553, Université de Savoie, 73376 Le Bourget-du-Lac Cedex, France;* [3]*Parc naturel régional Périgord-Limousin, La Barde, 24450 La Coquille, France; and* [4]*Laboratoire d'Ecologie Alpine, UMR CNRS 5553, Université Grenoble I, 38041 Grenoble Cedex 9, France*

Summary

1. Alien invasive species (AIS) are one of the major causes of biodiversity loss and global homogenization. Once an AIS becomes established, costs of control can be extremely high and complete eradication is not always achieved. The ability to detect a species at a low density greatly improves the success of eradication and decreases both the costs of control and the impact on ecosystems.

2. In this study, we compare the sensitivity of traditional field methods, based on auditory and visual encounter surveys, with an environmental DNA (eDNA) survey for the detection of the American bullfrog *Rana catesbeiana* = *Lithobates catesbeianus*, which is invasive in south-western France.

3. We demonstrate that the eDNA method is valuable for species detection and surpasses traditional amphibian survey methods in terms of sensitivity and sampling effort. The bullfrog was detected in 38 sites using the molecular method, compared with seven sites using the diurnal and nocturnal surveys, suggesting that traditional field surveys have strongly underestimated the distribution of the American bullfrog.

4. *Synthesis and applications*. The environmental DNA approach permits the early detection of alien invasive species (AIS), at very low densities and at any life stage, which is particularly important for the detection of rare and/or secretive aquatic species. This method can also be used to confirm the sensitivity of control operations and to better identify the distributions of vulnerable species, making this a very relevant tool for species inventory and management.

Key-words: alien invasive species, DNA barcoding, environmental DNA, inventory, *Lithobates catesbeianus*, species detection

Introduction

Alien invasive species (AIS) constitute one of major causes of biodiversity loss and global homogenization (Vitousek *et al.* 1997; Ficetola, Thuiller & Miaud 2007b; Ehrenfeld 2010; Pyšek & Richardson 2010). They may out-compete native species, act as predators or transmit exotic diseases. For example, in 1991, 68% of freshwater fishes in the continental United States known to have gone extinct since 1890 were negatively affected by introduced non-native fishes (Wilcove & Bean 1994). Once an AIS becomes established, costs of control action can be extremely high, complete eradication cannot always be achieved (Howald *et al.* 2007) and this may negatively affect the environment and compromise the recovery of native species (Myers *et al.* 2000).

During the early stages of AIS introduction, detection of the species is not possible unless its density exceeds a certain threshold (Hulme 2006; Harvey, Qureshi & MacIsaac 2009). The detection threshold depends on the monitoring method used and species detection may only be possible once the

*Correspondence author. E-mail: tony.dejean@spygen.fr

†Present address: Centre d'Ecologie Fonctionnelle et Evolutive (UMR 5175), Ecole Pratique des Hautes Etudes, Biogéographie et Ecologie des Vertébrés, campus CNRS, 1919 route de Mende, 34 293 Montpellier cedex 5, France.

species is already well-established (Myers *et al*. 2000). The ability to detect an AIS at low densities greatly determines the success of an eradication operation, decreases the costs of control and reduces the impact on ecosystems (Mehta *et al*. 2007). Therefore, there is an urgent need for methods that improve the probability of detection (Harvey, Qureshi & MacIsaac 2009).

Native to Eastern North America, the American bullfrog has been introduced worldwide (Lever 2003). It is considered to be one of the world's 100 worst invasive species (Lowe *et al*. 2000; D'Amore 2012). In Europe, bullfrogs have been introduced in at least 25 countries during the 20th century (Ficetola *et al*. 2007a). Three populations are successfully established in France (Fig. 1), and two of them are subject to control actions consisting of egg mass removal, tadpole trapping and shooting of juveniles and adults (Dejean 2008). In the population established in south-western France (Dordogne), bullfrogs were detected in 35 water bodies in 2006 using traditional survey techniques (calling and visual encounter surveys, Dodd 2010). Control actions started in 2006 and bullfrogs were detected in 19 aquatic sites in 2007 and seven sites in 2008 (Guibert, Dejean & Hippolyte 2010). Control actions seem to reduce the density of bullfrogs, but the relationship between amphibian detection probability and density suggests that small populations are more likely to escape detection (Tanadini & Schmidt 2011). The detection probability of low-density populations can be increased by increasing sampling effort (i.e. increasing the number of visit per site). In this study, we propose to use the newly developed environmental DNA (eDNA) barcoding approach (Hebert *et al*. 2003; Ficetola *et al*. 2008; Valentini, Pompanon & Taberlet 2009), using water samples as the DNA source. This method has been successfully implemented in the detection of invasive (Ficetola *et al*. 2008; Jerde *et al*. 2011) or secretive species (Goldberg *et al*. 2011). We compare and consider the potential errors of both methods (surveys and eDNA).

Fig. 1. Distribution of introduced bullfrog populations in France. 1: Colonized area in Gironde (introduction in 1968), 2: colonized region in Dordogne (introduction in 1990). This study was performed in this region (see Fig. 2). 3: colonized region in Loir et Cher (introduction suspected in the 2000). The comparisons of costs between traditional and environmental DNA (eDNA) surveys were performed in this area (see Discussion). (Map kindly provided by J. Lescure and J.-C. de Massary).

Materials and methods

STUDY AREA

The study was conducted within the Natural Regional Park of Perigord-Limousin, in the south-west of France (Fig. 2). About 80 aquatic sites were identified in the 5 × 5 km study area. The ponds are natural field depressions and old (*c*. 17th century) fishery ponds. The fishery activity ceased at the end of the 19th century. The Dronne river and its two tributaries (Fig. 2) are the only running water in this area.

BULLFROG DETECTION ESTIMATE

The presence of the American bullfrog was first detected in this region in the early 90s. To estimate detection probability of bullfrogs with traditional field survey methods, a study was conducted in eight ponds where bullfrog presence had been confirmed for several years (Dejean 2008). These ponds were sampled on four occasions (8, 20, 27 and 29 June 2006, methods described below). The weather conditions (temperature and wind) during these survey days were optimal for bullfrog activity in south-western France (Ficetola *et al*. 2007a).

Detection probability and site occupancy were modelled according to MacKenzie *et al*. (2006). We tested two models (detection probabilities were either constant or varied among the four sampling occasions) for each bullfrog category (juveniles, males and females) with the software PRESENCE 3.1 (MacKenzie *et al*. 2006). Evaluation of the relative performance of the models was based on Akaike Information Criterion (AIC) (Burnham & Anderson 2002), and models with lower AIC are considered better candidates than those with higher AIC.

ERADICATION PROGRAM

The staff of the PNR decided to implement an eradication program in 2005. Forty-nine ponds were selected (most of unselected sites within the study area were small ponds with temporary water, Dejean 2008), including the eight ponds described above, that were surveyed to determine bullfrog detection probability. The eradication methods included egg mass removal, tadpole trapping and shooting of juveniles and adults (Dejean 2008). In late spring and summer 2006, 601 bullfrogs were detected and shot in 32 ponds, of these 515 were juveniles, 74 were males and 12 were females. In 2007, 412 individuals were shot in 19 ponds, including 339 juveniles, 59 males and 14 females. In 2008, 334 individuals were shot in seven ponds, including 313 juveniles, 14 males and seven females (Guibert, Dejean & Hippolyte 2010).

TRADITIONAL FIELD SURVEYS

Surveys were performed in 2006 (eight ponds, see above for methods) and 2008 (49 ponds, from 16 to 20 June) with the help of skilled herpetologists: (i) During the day, a visual encounter survey was performed on the borders of each pond (up to 1 h depending on the pond size): the shoreline was followed and investigated using binoculars, to detect adults and juveniles. The

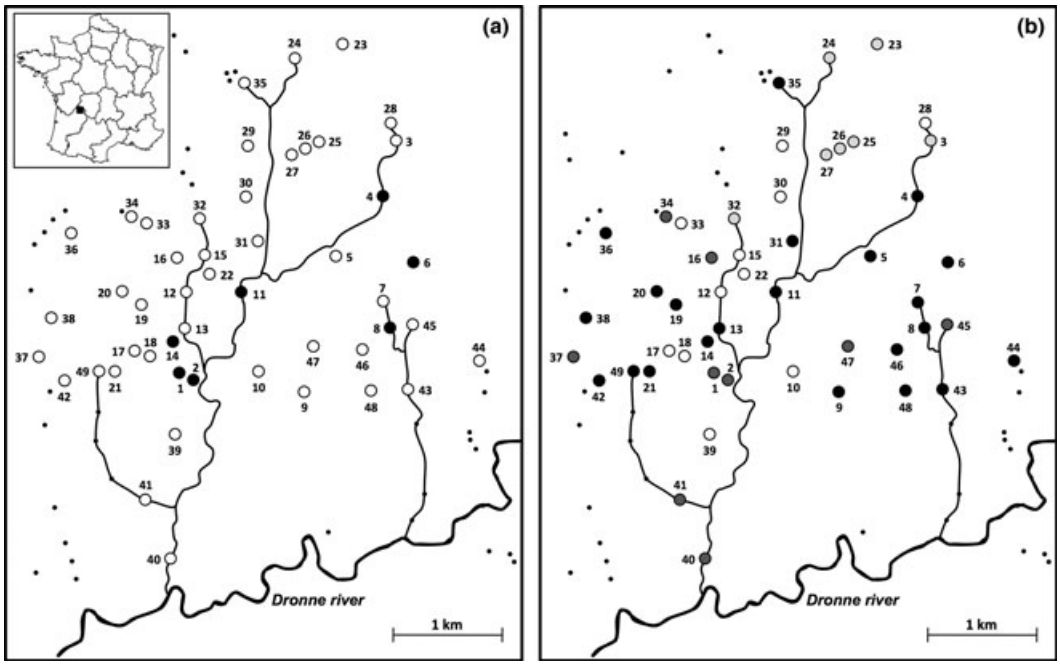

Fig. 2. Distribution of bullfrog with traditional surveys (a) filled circles represent the ponds where the American bullfrog was detected, and open circles represent the pond where the species was not detected. Distribution of bullfrog with environmental DNA (eDNA) survey (b) filled light grey circles represent the ponds where the American bullfrog was detected in one of the three water samples, filled dark grey circles represent the ponds where it was detected in two of the three water samples, filled black circles represent the ponds where it was detected in all the water samples and open circles represent the pond where the species was not detected. Dots represent the ponds that were not surveyed. This study area refers to the colonized region 2 in Fig. 1.

water near the shoreline was also searched for egg masses and tadpoles. (ii) From about 10:00 PM (sunset) to 02:00 AM, the ponds were visited again, and a calling survey was performed. The ponds were approached quietly to a distance of about 50 m from the pond edge. Upon reaching the survey position, the surveyor waited for 5 min before carrying out the auditory survey. Each survey was carried out for a maximum of 15 min and was concluded as soon as a bullfrog call was detected.

eDNA SURVEY

The sampling strategy used for bullfrog detection with eDNA followed the protocol as described by Ficetola *et al.* (2008). Sampling occurred in the same time period as the traditional survey (16–20 June 2008), and three 15-mL water samples were collected from different sections of each pond where the species was most likely present (i.e. mainly in areas rich in aquatic vegetation). For each pond, the samples were collected the same day. Immediately after collection, a solution composed of 1·5 mL of sodium acetate 3 M and 33 mL absolute ethanol was added to the water samples and the tubes were then stored at −20 °C until DNA extraction. A total of 147 water samples were collected from 49 ponds. All samples for the eDNA survey were composed by clear water.

DNA extraction and amplification methods were adapted from Ficetola *et al.* (2008). The mixture was centrifuged at 9400 g for 1 h at 6 °C to recover DNA and/or cellular remains. The DNA from the resulting pellet was extracted using QIAmp Blood and Tissue Extraction Kit (GmbH; Qiagen, Hilden, Germany), following manufacturer's instructions. DNA extraction was performed in a room dedicated for degraded DNA samples. Control extractions were systematically performed to monitor possible contaminations. Bullfrog DNA was amplified with specific primers (Ficetola *et al.* 2008). DNA amplifications were carried out in a final volume of 25 μL, using 3 μL of DNA extract as template. Three PCR replicates were performed per DNA sample. The amplification mixture contained 1 U of Ampli*Taq* Gold DNA Polymerase (Applied Biosystems, Foster City, CA, USA), 10 mM Tris-HCl, 50 mm KCl, 2 mM of MgCl2, 0·2 mM of each dNTPs, 0·2 μm of each primer and 0·005 mg of bovine serum albumin (BSA; Roche Diagnostics, Basel, Switzerland). After 10 min at 95 °C (*Taq* activation), the PCR cycles were performed as follows: 55 cycles of 30 s at 95 °C, 30 s at 61 °C. PCR products were visualized using electrophoresis on 2% agarose gel. Negative (UHQ water) as well as positive samples (DNA extracted from American bullfrog tissue) were systematically added during the PCR step.

Results

TRADITIONAL FIELD SURVEYS

The models with constant detection probability provided a better explanation of observed survey data collected in 2006 for each bullfrog category (Table 1): the detection probability was 0·80 ± 0·073 in juveniles, 0·73 ± 0·080 in males and 0·27 ± 0·081 in females, respectively.

The presence of bullfrogs was detected in seven of the 49 ponds surveyed in 2008 (occurrence = 0·143). Tadpoles were observed during diurnal visual encounter surveys in three ponds while males were detected by nocturnal calling surveys in four other ponds (Fig. 1a and Table 2).

Table 1. Model selection for bullfrog detection probability inference using traditional visual methods (encounter and calling surveys) for amphibian detection

	Model	AIC	Model likelihood	Nb parameters
Juveniles	*P* constant	34·02	1·00	2
	P variable	34·99	0·62	5
Males	*P* constant	38·79	1·00	2
	P variable	43·25	0·11	5
Females	*P* constant	38·79	1·00	2
	P variable	38·81	0·99	5

Aic, Akaike Information Criterion; Nb, number.

eDNA SURVEY

Bullfrog eDNA was successfully amplified in water samples collected in 38 out of 49 ponds. Positive bullfrog DNA signal was obtained for all three replicates in 22 ponds, for two of the three replicates in nine ponds and for one of the three replicates in seven ponds. Three PCR replicates were performed for each DNA extract (i.e. a total of nine PCRs for each pond). The overall average bullfrog DNA amplification success was 0·53 ± 0·03 (min: 1/3; max: 3/3, $N = 38$). The eDNA survey produced a bullfrog occurrence of 0·775 (Fig. 1b and Table 2).

COMPARING TRADITIONAL AND eDNA SURVEYS

Positive results were obtained for the eDNA survey from all seven ponds where bullfrogs were detected using traditional survey methods (Table 2). The amplification success in these ponds was 0·62 ± 0·06 (min: 3/9; max: 8/9, $N = 7$). It is not possible to infer relation between eDNA amplification success and bullfrog density because the surveys were not designed to estimate this parameter.

Excluding these seven ponds as well as the 11 ponds where bullfrogs were not detected (using both traditional and eDNA surveys), the amplification success was 0·52 ± 0·03 (min: 1/9; max: 9/9) in the remaining 31 ponds.

No bullfrog presence was detected in pond number #41 from using the traditional survey method, whereas two of three water samples and five of nine PCRs were positives (Table 2). Similarly, four ponds (#13, 19, 20 and 21) showed amplification of bullfrog DNA in all water sample replicates (seven of nine positive PCRs) while no bullfrog presence was detected at these ponds using traditional survey methods.

Overall, the eDNA method indicated bullfrog occurrence in 38 out of 49 ponds (0·775) which is more than five times higher than that found in traditional surveys (seven out of 49; 0·143).

Table 2. Detection of bullfrog using traditional and environmental DNA (eDNA) surveys (June 16–20, 2008)

	Traditional surveys		eDNA surveys		
Pond	Visual encounter detection	Calling detection	Detection	Water samples positives	Positive PCRs
1	+		+	2/3	6/9
2		+	+	2/3	6/9
3			+	1/3	1/9
4		+	+	3/3	3/9
5			+	3/3	8/9
6	+		+	3/3	9/9
7			+	3/3	6/9
8		+	+	3/3	3/9
9			+	3/3	6/9
10				0/3	0/9
11	+		+	3/3	4/9
12				0/3	0/9
13			+	3/3	7/9
14		+	+	3/3	8/9
15				0/3	0/9
16			+	2/3	3/9
17				0/3	0/9
18				0/3	0/9
19			+	3/3	7/9
20			+	3/3	7/9
21			+	3/3	7/9
22				0/3	0/9
23			+	1/3	2/9
24			+	1/3	2/9
25			+	1/3	1/9
26			+	1/3	1/9
27			+	1/3	2/9
28				0/3	0/9
29				0/3	0/9
30				0/3	0/9
31			+	3/3	9/9
32			+	1/3	1/9
33				0/3	0/9
34			+	2/3	2/9
35			+	3/3	6/9
36			+	3/3	4/9
37			+	2/3	6/9
38			+	3/3	8/9
39				0/3	0/9
40			+	2/3	3/9
41			+	2/3	5/9
42			+	3/3	4/9
43			+	3/3	4/9
44			+	3/3	6/9
45			+	2/3	3/9
46			+	3/3	6/9
47			+	2/3	2/9
48			+	3/3	8/9
49			+	3/3	9/9

Discussion

The precise understanding of species distribution is a key requirement for conservation management, especially when the focal species is invasive (Magurran 2003; Harvey, Qureshi & MacIsaac 2009). The ability to detect a species at a low density greatly influences management decisions (Mehta *et al.* 2007), making the development of methods to improve detection probabilities a high priority. In this study, we take advantage of a control action to manage the invasive American bullfrog in the Natural Regional Park of Périgord-Limousin to compare traditional field surveys and eDNA surveys. The control actions performed from 2006 onwards were considered as effective because 35 water bodies were detected as colonized in

2006, 19 in 2007 and seven in 2008 (Guibert, Dejean & Hippolyte 2010). However, if the observed trend in occurrence is correlated with a population size decrease, there is a high risk of overestimating the success of this action because of the well-known relationship between detection and density in amphibians (e.g. Tanadini & Schmidt 2011).

The eDNA methods constitute a promising tool in ecology (Ficetola *et al.* 2008); however, it is important to assess the efficiency by comparing different survey methods to determine their relative reliabilities.

BULLFROG DETECTION COMPARED WITH TRADITIONAL SURVEY

The reliability of amphibian surveys, as any other species detection method, can be compromised by the possibility of false positives (Type I errors, species is detected where it is not present) and/or false negatives (Type II errors, species is not detected where it is present). Bullfrog detection was based on calls, tadpoles and spawns determination. This species is easy to identify among the other native amphibians of the studied area, and we consider that the possibility of false positives is closed to null.

On the other hand, a bullfrog not detected where it is present is a classical issue in amphibian population monitoring (see Dodd 2010 for a review). Amphibian occurrence or abundance strongly depends on species detectability, which vary with many factors; these include the date and time of day, meteorological conditions, population size and observer experience (Crouch & Paton 2002; Genet & Sargent 2003; Schmidt & Pellet 2010; Tanadini & Schmidt 2011).

Calling surveys are one of the most popular methods (e.g. Pellet & Schmidt 2005; de Solla *et al.* 2005; Weir *et al.* 2005), because male vocalizations to attract females are easily identifiable, making their detection and identification relatively simple when weather conditions are favourable (e.g. Pellet & Schmidt 2005). In the Périgord-Limousin region, bullfrog detectability (combining calling and visual encounter surveys) was similar and rather high for juveniles and males ($0{\cdot}80 \pm 0{\cdot}073$ and $0{\cdot}73 \pm 0{\cdot}080$, respectively) while it decreased to $0{\cdot}27 \pm 0{\cdot}081$ for females. The reproductive biology and behavioural differences between life stages can explain this result (e.g. no calling activity and territory defence in females, Ryan 1980). Pellet & Schmidt 2005 showed that for the common toad (*Bufo bufo*), a pond-breeding amphibian, at least six visits (15 min each) were necessary to infer the presence of the species with 95% confidence. If the decrease in bullfrog density from 2006 to 2008 leads to a reduced detection probability, this phenomenon can be counterbalanced by an increased sampling effort (i.e. the number of visit per site). The large difference in bullfrog occurrence estimates in 2008 between the two methods (traditional vs. eDNA) suggested that the bullfrog distribution was underestimated using traditional field surveys. Following this result, the staff of the PNR Périgord-Limousin was highly motivated to perform a new field survey (more than five visits per site) on the 38 ponds identified as bullfrog positive with eDNA. Bullfrogs were detected in the previous seven ponds, and in 11 'new' water bodies. The estimated occurrence of bullfrogs thus reached 0·47 with this strong sampling effort, in a set of ponds that the eDNA method identified as bullfrog positive. Goldberg *et al.* (2011) demonstrated the sensitivity of the eDNA method in a case study involving Asian carps in North America, by comparing it with classical electrofishing. At the lowest carp density, only eDNA was able to detect carp presence. One carp was detected in one pool after 93 person-days of electrofishing effort that was motivated and targeted by the discovery of carp eDNA.

DETECTION PROBABILITY WITH eDNA SURVEY

The reliability of the genetic method can be compromised by the possibility of false positives and/or negatives (Type I and II errors, see above), that is, non-specificity of the primers used for DNA amplification, contamination or protracted DNA persistence after the death of the organism, poor sampling or poor protocol efficiency (Darling & Mahon 2011). The protocol used had been shown to be reliable in a previous study (Ficetola *et al.* 2008): before the eDNA analysis, primer reliability, robustness and specificity were tested, first *in silico* [using ECOPCR software (Taberlet *et al.* 2007)] and then on high-quality DNA (extracted from tissues samples), and PCR conditions were optimized (Ficetola *et al.* 2008). Because of the rarity of DNA in the water samples, the analysis was performed with similar precautions as those used for ancient DNA studies to reduce contamination and poor-quality DNA results (Taberlet *et al.* 1996; Cooper & Poinar 2000). This means that DNA was extracted in a dedicated room for rare DNA, mock samples without DNA and positive samples were analysed in parallel, the number of PCR cycles was increased, the analysis was performed on several field samples and three PCR replicates per sample were performed. Based on three samples per pond, the amplification success was $0{\cdot}37 \pm 0{\cdot}1$ in ponds where bullfrogs were present at low densities and $0{\cdot}79 \pm 0{\cdot}08$ in ponds where bullfrogs were present at high densities (Ficetola *et al.* 2008). In the present study, this amplification rate was $0{\cdot}53 \pm 0{\cdot}03$. It is too early to infer quantitative (i.e. abundance or density) information from eDNA survey results, but the amplification rate could be useful to that end in the future.

Several ponds within the study area cannot be considered to be discrete units, because they are connected by small streams (Fig. 1). It is thus possible that DNA moves from one site to another, leading to false positives (bullfrog eDNA detected in bullfrog free pond). However, in our study, eDNA revealed the presence of bullfrog in ponds 32 and 13 (about 1 km apart, Fig. 1), and not in ponds 12 and 15 located between them on the same tributary. Note that there is no water flow in the stream at this time of year, which limits eDNA dispersal.

Finally, bullfrogs can move from one pond to another, leaving eDNA in an unoccupied pond at the time of sampling, which will lead in turn to false positives. The mean distance between adjacent ponds in the sampling area is lower than adult bullfrog potential dispersal (Ingram & Raney 1943; Willis, Moyle & Baskett 1956). However, adult and juvenile

dispersal inferred with radiotracking and pit-fall trap methods in south-western France (Berroneau, Detaint & Coïc 2007) showed that both life stages stayed in aquatic sites during the June study period. In addition, the persistence of eDNA in water has been tested (Dejean *et al.* 2011) and bullfrog eDNA was detected only for a maximum of 2 weeks after the removal of the source animal. It is thus highly probable the bullfrog was present or had been present at maximum 2 weeks prior to the time the water sampling was performed, which further limits the possibility of type I errors.

The use of eDNA as a survey tool in ecology is in a developmental phase. The reliable detection of aquatic vertebrates was confirmed in wetlands (Ficetola *et al.* 2008), in a large river and canal system (study of the risk of invasion of the Laurentian Great Lakes region by Asian Carp, Jerde *et al.* 2011) and streams (inventory of secretive Rocky Mountain tailed frogs, *Ascaphus montanus*, and Idaho giant salamanders, *Dicamptodon aterrimus*, in the north-western region of the United States, Goldberg *et al.* 2011). These studies show higher detectability for rare and/or secretive aquatic species, at all life stages and at low densities. In pond-breeding amphibians (this study), detection with traditional methods is very sensitive to meteorological conditions and often limited in the season (short stay in water of breeding adults). Water sampling for eDNA can be performed whatever the weather conditions and for a longer period of time because more cryptic individuals (e.g. tadpoles) stay in the water. In another introduced bullfrog population (32 colonized ponds, Loir et Cher, Central France, Fig. 1), a preliminary estimate of traditional and eDNA survey costs showed that the eDNA method was 2·5 times cheaper and 2·5 times less time consuming than the traditional survey (based on a 2-person fieldwork time and complete molecular analysis) (Michelin, Heckly & Rigaux 2011).

The sensitivity of eDNA across taxa and environments remains to be determined. The method is efficient for vertebrate inventory in freshwater environments, with rare and/or secretive species, at low densities and at several life stages. This method is particularly promising for other taxonomic groups (e.g. microorganisms, plants) and other environments (e.g. soils).

Acknowledgements

This study was supported by the EU (FEDER Limousin), the Limousin Region, the Rhône-Alpes Region (programme CIBLE) and the Association Nationale pour la Recherche Technique (ANRT). TD was supported by a PhD scholarship funded by the Parc Naturel Régional Périgord-Limousin and CM by a EU Marie Curie grant (MOIF-CT-2006-022190, INTEGRINVA). We thank Sébastien Guibert and Nathalie Tissot for their help in, respectively, the field and the laboratory. We also thank Gabriel Michelin, Dominique Beguin, Mathieu Berroneau, Christophe Coïc, Catherine Epain-Henry, Olivier Lorvelec and Fabienne Renard-Laval for their implication in the knowledge and control of bullfrogs in France and Dr F. Ficetola and an anonymous reviewer for very useful and constructive comments.

References

Berroneau, M., Detaint, M. & Coïc, C. (2007) Premiers résultats du suivi radio télémétrique de la Grenouille taureau en Gironde (septembre 2004–juin 2005). *Bulletin de la Société herpétologique de France*, **121**, 21–33.

Burnham, K.P. & Anderson, D.R. (2002) *Model Selection and Multimodel Inference: A Practical Information-Theoretic Approach*. Springer Verlag, New York.

Cooper, A. & Poinar, H.N. (2000) Ancient DNA: do it right or not at all. *Science*, **289**, 1139.

Crouch, W.B. & Paton, P.W.C. (2002) Assessing the use of call surveys to monitor breeding anurans in Rhode Island. *Journal of Herpetology*, **36**, 185–192.

D'Amore, A. (2012) *Rana (Lithobates) catesbeiana* Shaw (American Bullfrog). *Handbook of Global Freshwater Invasive Species* (ed R. Francis), pp. 321–330. Earthscan, Taylor & Francis Group, Abingdon, UK.

Darling, J.A. & Mahon, A.R. (2011) From molecules to management: adopting DNA-based methods for monitoring biological invasions in aquatic environments. *Environmental Research*, **111**, 978–988.

Dejean, T. (2008) *Opération pilote d'éradication de la Grenouille taureau*. Rapport final, Parc naturel régional Périgord-Limousin, Abjat-sur-Bandiat.

Dejean, T., Valentini, A., Duparc, A., Pellier-Cuit, S., Pompanon, F., Taberlet, P. & Miaud, C. (2011) Persistence of environmental DNA in freshwater ecosystems. *PLoS One*, **6**, e23398.

Dodd, C.K.J. (2010) *Amphibian Ecology and Conservation: A Handbook of Techniques*, Oxford University Press. C. Kenneth Dodd Jr., Oxford.

Ehrenfeld, J.G. (2010) Ecosystem consequences of biological invasions. *Annual Review of Ecology, Evolution, and Systematics*, **41**, 59–80.

Ficetola, G.F., Thuiller, W. & Miaud, C. (2007b) Prediction and validation of the potential global distribution of a problematic alien invasive species-the American bullfrog. *Diversity and Distributions*, **13**, 476–485.

Ficetola, G.F., Coïc, C., Detaint, M., Berroneau, M., Lorvelec, O. & Miaud, C. (2007a) Pattern of distribution of the American bullfrog *Rana catesbeiana* in Europe. *Biological Invasions*, **9**, 767–772.

Ficetola, G.F., Miaud, C., Pompanon, F. & Taberlet, P. (2008) Species detection using environmental DNA from water samples. *Biology Letters*, **4**, 423.

Genet, K.S. & Sargent, L.G. (2003) Evaluation of methods and data quality from a volunteer-based amphibian call survey. *Wildlife Society Bulletin*, **31**, 703–714.

Goldberg, C.S., Pilliod, D.S., Arkle, R.S. & Waits, L.P. (2011) Molecular detection of vertebrates in stream water: a demonstration using rocky mountain tailed frogs and Idaho giant salamanders. *PLoS One*, **6**, e22746.

Guibert, S., Dejean, T. & Hippolyte, S. (2010) Le Parc naturel régional Périgord-Limousin: territoire d'expérimentation et d'innovation par la mise en place d'un programme d'éradication de la Grenouille taureau (Lithobates catesbeianus) associé à un programme de recherche sur les maladies émergentes des amphibiens. *Epops*, **1**, 5–24.

Harvey, C.T., Qureshi, S.A. & MacIsaac, H.J. (2009) Detection of a colonizing, aquatic, non-indigenous species. *Diversity and Distributions*, **15**, 429–437.

Hebert, P.D., Cywinska, A., Ball, S.L. & DeWaard, J.R. (2003) Biological identifications through DNA barcodes. *Proceedings of the Royal Society of London. Series B: Biological Sciences*, **270**, 313.

Howald, G., Donlan, C., Galván, J.P., Russell, J.C., Parkes, J., Samaniego, A., Wang, Y., Veitch, D., Genovesi, P., Pascal, M., Saunders, A. & Tershy, B. (2007) Invasive rodent eradication on islands. *Conservation Biology*, **21**, 1258–1268.

Hulme, P.E. (2006) Beyond control: wider implications for the management of biological invasions. *Journal of Applied Ecology*, **43**, 835–847.

Ingram, W.M. & Raney, E.C. (1943) Additional studies on the movement of tagged bullfrogs, Rana catesbeiana Shaw. *American Midland Naturalist*, **29**, 239–241.

Jerde, C.L., Mahon, A.R., Chadderton, W.L. & Lodge, D.M. (2011) « Sight-unseen » detection of rare aquatic species using environmental DNA. *Conservation Letters*, **4**, 150–157.

Lever, C. (2003) *Naturalized Reptiles and Amphibians of the World*. Oxford University Press, New York.

Lowe, S., Browne, M., Boudjelas, S. & De Poorter, M. (2000) 100 of the world's worst invasive alien species a selection from the global invasive species database. Published by The Invasive Species Specialist Group (ISSG) a specialist group of the Species Survival Commission (SSC) of the World Conservation Union (IUCN), 12pp. First published as special lift-out in Aliens, **12**.

MacKenzie, D.I., Nichols, J.D., Royle, J.A., Pollock, K.H., Bailey, L.L. & Hines, J.E. (2006) *Occupancy Estimation and Modeling: Inferring Patterns and Dynamics of Species Occurrence*. Academic Press, New York, USA.

Magurran, A.E. (2003) *Measuring Biological Diversity*, 1st edn. Wiley-Blackwell, Oxford.

Mehta, S.V., Haight, R.G., Homans, F.R., Polasky, S. & Venette, R.C. (2007) Optimal detection and control strategies for invasive species management. *Ecological Economics*, **61**, 237–245.

Michelin, G., Heckly, X. & Rigaux, B. (2011) *Rapport d'étude – ADN Environnemental, Détection de l'Espèce Exotique Envahissante Grenouille Taureau*, pp. 20. DREAL, CDPNE, CR Centre, Spygen.

Myers, J.H., Simberloff, D., Kuris, A.M. & Carey, J.R. (2000) Eradication revisited: dealing with exotic species. *Trends in Ecology & Evolution*, **15**, 316–320.

Pellet, J. & Schmidt, B.R. (2005) Monitoring distributions using call surveys: estimating site occupancy, detection probabilities and inferring absence. *Biological Conservation*, **123**, 27–35.

Pyšek, P. & Richardson, D.M. (2010) Invasive species, environmental change and management, and health. *Annual Review of Environment and Resources*, **35**, 25.

Ryan, M.J. (1980) The reproductive behavior of the bullfrog (*Rana catesbeiana*). *Copeia*, **1**, 108–114.

Schmidt, B.R. & Pellet, J. (2010) Quantifying abundance: counts, detection probabilities, and estimates. *Amphibian Ecology and Conservation: A Handbook of Techniques* (ed. C.K. Dodd Jr), pp. 465–479. Oxford University Press, Oxford.

de Solla, S.R., Shirose, L.J., Fernie, K.J., Barrett, G.C., Brousseau, C.S. & Bishop, C.A. (2005) Effect of sampling effort and species detectability on volunteer based anuran monitoring programs. *Biological Conservation*, **121**, 585–594.

Taberlet, P., Griffin, S., Goossens, B., Questiau, S., Manceau, V., Escaravage, N., Waits, L.P. & Bouvet, J. (1996) Reliable genotyping of samples with very low DNA quantities using PCR. *Nucleic Acids Research*, **24**, 3189.

Taberlet, P., Coissac, E., Pompanon, F., Gielly, L., Miquel, C., Valentini, A., Vermat, T., Corthier, G., Brochmann, C. & Willerslev, E. (2007) Power and limitations of the chloroplast trnL (UAA) intron for plant DNA barcoding. *Nucleic Acids Research*, **35**, e14.

Tanadini, L.G. & Schmidt, B.R. (2011) Population size influences amphibian detection probability: implications for biodiversity monitoring programs. *PLoS One*, **6**, e28244.

Valentini, A., Pompanon, F. & Taberlet, P. (2009) DNA barcoding for ecologists. *Trends in Ecology & Evolution*, **24**, 110–117.

Vitousek, P.M., D'antonio, C.M., Loope, L.L., Rejmanek, M. & Westbrooks, R. (1997) Introduced species: a significant component of human-caused global change. *New Zealand Journal of Ecology*, **21**, 1–16.

Weir, L.A., Royle, J.A., Nanjappa, P. & Jung, R.E. (2005) Modeling anuran detection and site occupancy on North American Amphibian Monitoring Program (NAAMP) routes in Maryland. *Journal of Herpetology*, **39**, 627–639.

Wilcove, D.S. & Bean, M.J. (1994) *The Big Kill: Declining Biodiversity in America's Lakes and Rivers*. Environmental Defense Fund, Washington, District of Columbia, USA.

Willis, Y.L., Moyle, D.L. & Baskett, T.S. (1956) Emergence, breeding, hibernation, movements and transformation of the bullfrog, Rana catesbeiana, in Missouri. *Copeia*, **1**, 30–41.

Received 4 January 2012; accepted 7 June 2012
Handling Editor: Chris Frid