Received: 1 February 2019 | Revised: 13 June 2019 | Accepted: 17 June 2019

DOI: 10.1111/csp2.86

CONTRIBUTED PAPER

Conservation Science and Practice
A journal of the Society for Conservation Biology
Open Access

WILEY

Early detection and spatial monitoring of an emerging biological invasion by population genetics and environmental DNA metabarcoding

Christophe Dufresnes[1,2] | **Tony Déjean**[3] | **Silvia Zumbach**[4] | **Benedikt R. Schmidt**[4,5] | **Luca Fumagalli**[2] | **Petra Ramseier**[6] | **Sylvain Dubey**[1,2,7]

[1]Hintermann & Weber SA, Montreux, Switzerland

[2]Laboratory for Conservation Biology, Department of Ecology and Evolution, University of Lausanne, Lausanne, Switzerland

[3]SPYGEN, Le Bourget-du-Lac, France

[4]Info fauna—karch, UNiMail, Neuchâtel, Switzerland

[5]Institut für Evolutionsbiologie und Umweltwissenschaften, Universität Zürich, Zürich, Switzerland

[6]Hintermann & Weber AG, Reinach, Switzerland

[7]Agrosustain SA, Nyon, Switzerland

Correspondence
Christophe Dufresnes, Hintermann & Weber SA, Montreux, Switzerland.
Email: christophe.dufresnes@hotmail.fr

Funding information
Abteilung Naturförderung of canton Bern; Swiss Federal Office for the Environment (FOEN/OFEV/BAFU)

Abstract

Rapid management responses against invasive species soon after their establishment are the most efficient way to limit their biological and economic impacts. Early detection and reliable monitoring is however challenging when cryptic taxa are involved. Here we show how environmental DNA (eDNA) metabarcoding of water samples efficiently unveiled an emerging invasion of Italian crested newts (*Triturus carnifex*), one of the most successful amphibian invaders in Europe. We compared and validated an eDNA survey by multilocus population genetics of wild-caught individuals. Both approaches consistently mapped a localized *T. carnifex* invasion in northwestern Switzerland, most likely following imports from the Italian Po Plain. We found evidence of gene flow with the indigenous and endangered *Triturus cristatus* in nearby populations, suggesting a potential expansion. Yet the currently small invasive range should be efficiently contained by future eradication programs. This textbook case emphasizes the implementation of eDNA metabarcoding to screen aquatic communities for exotic species, from which targeted studies can be designed on emerging biological invasions.

KEYWORDS

amphibian, conservation genetics, environmental DNA, invasion monitoring, newt, *Triturus carnifex*

1 | INTRODUCTION

Invasive species are among the worst threats to biodiversity, with detrimental effects on native biota such as competition, predation, transmission of diseases, and genetic pollution through hybridization (Doherty, Glen, Nimmo, Ritchie, & Dickman, 2016; Lowry et al., 2013; Pysek & Richardson, 2010). For instance, nonnative invaders contributed to ~40% of all known animal extinctions since the 17th century (Secretariat of the Convention on Biological Diversity, 2006; up to 58% in amniotes, Doherty et al., 2016), and presently threaten ~42% of the red-listed species of the United States (Pimentel, Zuniga, & Morrison, 2005). Beyond the biological costs to our ecosystems, managing invasive species also consumes a significant portion of the budget available to conservation authorities (Hulme et al., 2008; Pimentel et al., 2005). These costs could be significantly reduced, and chances for successful elimination increased, if invaders were eradicated early, that is, before they establish expanding populations (Keller, Lodge, &

Finnoff, 2007). The early detection of invasive species is particularly challenging when they are ecologically and morphologically similar to native conspecifics, since wildlife professionals cannot reliably identify them on the field (Dubey, Lavanchy, Thiébaud, & Dufresnes, 2019). Molecular methods are a powerful tool to detect, describe, and quantify such cryptic invasions, although many alien species are already too widespread upon detection for complete eradications (e.g., Dufresnes et al., 2017).

Environmental DNA (eDNA) analysis may be a game-changer (Mahon & Jerde, 2016; Taberlet, Bonin, Zinger, & Coissac, 2018). Recently, eDNA methods have been developed to target some of the most harmful aquatic invaders such as in crayfish (Tréguier et al., 2014), molluscs (Clusa, Miralles, Basanta, Escot, & Garcia-Vazquez, 2017), mosquitoes (Schneider et al., 2016), fishes (Nevers et al., 2018), pathogenic fungi (Schmidt, Kéry, Ursenbacher, Hyman, & Collins, 2013), amphibians (Dejean et al., 2012), and plants (Gantz, Renshaw, Erickson, Lodge, & Egan, 2018). In parallel, the multispecies approach offered by eDNA metabarcoding may transform the monitoring of aquatic biodiversity (Deiner et al., 2017; Pont et al., 2018; Valentini et al., 2016), but its utility for the detection of emerging cryptic aliens faces several challenges. First, the taxonomic resolution of metabarcoding may not discriminate closely related taxa, given the aim to cover broad taxonomic groups using short conserved markers. Second, reference databases are often geographically and taxonomically biased, complicating the discrimination of unreferenced intraspecific variation. Third, the mitochondrial nature of markers used for eDNA metabarcoding prevents assessments of hybridization, which often occurs during aquatic invasion (Dufresnes et al., 2016; Fukumoto, Ushimaru, & Minamoto, 2015). Last but not least, the risk of false-positive and false-negative detections still remains a major challenge in eDNA methods (Darling & Mahon, 2011; Lahoz-Montfort, Guillera-Arroita, & Tingley, 2016; Schmidt et al., 2013). All these issues are highly relevant for efficiently monitoring biological invasions, which often involve cryptic species with low initial densities, introduced within the range of closely related hybridizing taxa. Hence, the use of eDNA to efficiently monitor invasive species would benefit from comparative assessments with classic invasion genetic methods.

Here we provide a demonstration with the case of the Italian crested newt (*Triturus carnifex*), one of the most successful invasive amphibians in Europe, by a combination of eDNA metabarcoding and genotyping of captured individuals. The Italian crested newt was translocated multiple times north of the Alps in Switzerland, Germany, the United Kingdom, and the Netherlands, within the range of the threatened Northern Crested newt (*Triturus cristatus*) (Fahrbach & Gerlach, 2018). *Triturus carnifex* is replacing *T. cristatus* through competition and introgressive hybridization (Dufresnes et al., 2016). Several of these invasions became country-wide issues, given the extent of their ranges and uncontrolled expansions that led to massive genetic replacement.

Crested newts show only subtle differences in coloration and morphology among cryptic species (Dufresnes, 2019; Fahrbach & Gerlach, 2018) and cannot be reliably identified without genetic tools. The presence of the Italian taxon was recently suspected from a pond system in a nature reserve south of Basel, Switzerland, based on photographic evidence (taken by N. Martinez in 2012, and identified by S.D. in 2016) and confirmed by preliminary eDNA data (see below). The question arises whether this new *T. carnifex* invasion can be contained or has already expanded and massively admixed with local *T. cristatus*, upon which management responses will depend. In parallel, this system offers an empirical opportunity to use eDNA as a surveillance tool of biological invasions when it involves closely related taxa and hybridization, for comparison with individual-based multilocus genetic monitoring.

To this end, we conducted a comparative survey combining eDNA metabarcoding with nuclear and mitochondrial population genetics of field-caught newts across the entire Basel area. Our objectives were to (a) document the origin and the spatial extent of this new invasion to inform relevant authorities and (b) to assess the performance of eDNA compared to multilocus inferences on wild-caught newts. Specifically, we test whether both approaches yield similar patterns of exotic versus autochthonous species distributions, as expected if eDNA metabarcoding efficiently detects and discriminates between lineages, despite the unknown origin of *T. carnifex*. Alternatively, discrepancies may arise because of the dynamics and nature of the emerging invaders, for example, due to low density, unreferenced sources, and potential cyto-nuclear discordances caused by hybridization.

2 | METHODS

2.1 | eDNA metabarcoding survey

In 2017, water samples from five ponds located in the surroundings of Basel (cantons of Basel-Stadt, hereafter BS, and Basel-Landschaft, hereafter BL; File S1) were analyzed using the SPYGEN eDNA metabarcoding technology (SPYGEN, Le Bourget-du-Lac, France). In 2018, we sampled three of them again, and extended the survey to 17 additional sites. A total of 22 sites were thus analyzed over both years.

The field survey methodology was modified from Biggs et al. (2015), using a single sampling kit comprising a sterile

water sampling ladle, a self-supporting sterile Whirl-Pak bag, a sterile syringe, gloves to minimize contamination, a VigiDNA 0.45-μM cross-flow filtration capsule (SPYGEN, Le Bourget-du-Lac, France) and 80 mL bottle of CL1 Conservation buffer (SPYGEN, Le Bourget-du-Lac, France). Samples of 100 mL water were collected with the ladle around the pond margin, totaling 2 L per site. Collectors did not enter the water to avoid possible contamination or by stirring up sediment. Samples were homogenized by gentle shaking, filtered through the capsule, and preserved at room temperature with the conservation buffer.

DNA extraction was performed following the protocol described by Pont et al. (2018) in a dedicated room for water DNA sample extraction, equipped with positive air pressure, ultraviolet treatment, and frequent air renewal. Before entering this extraction room, personnel changed into full protective clothing comprising a disposable body suit with hood, mask, laboratory shoes, overshoes, and gloves in a connecting zone. All benches were decontaminated with 10% commercial bleach before and after each manipulation. For DNA extraction, each filtration capsule, containing the CL1 buffer, was agitated for 15 min on an S50 shaker (IngenieurBüro CAT, Ballrechten-Dottingen, Germany) at 800 rpm. The buffer was then emptied into a 50 mL tube, before being centrifuged for 15 min at 15,000 g. The supernatant was removed with a sterile pipette, leaving 15 mL of liquid at the bottom of the tube. Subsequently, 33 mL of ethanol and 1.5 mL of 3 M sodium acetate were added to each 50 mL tube and stored for at least one night at −20°C. The tubes were centrifuged at 15,000 *g* for 15 min at 6°C, and the supernatants were discarded. After this step, 720 μL of ATL buffer from the DNeasy Blood and Tissue Extraction Kit (Qiagen) was added. The tubes were then vortexed, and the supernatants were transferred to 2 mL tubes containing 20 μL of proteinase K. The tubes were finally incubated at 56°C for 2 hr. Subsequently, DNA extraction was performed using NucleoSpin Soil (Macherey-Nagel GmbH & Co., Düren, Germany) starting from step 6 and following the manufacturer's instructions. The elution was performed by adding 100 μL of SE buffer twice. After the DNA extraction the samples were tested for inhibition by quantitative polymerase chain reaction (PCR) (Biggs et al., 2015). If the sample was considered inhibited, it was diluted fivefold before the amplification.

DNA amplifications were performed in a final volume of 25 μL, using 3 μL of DNA extract as template. The amplification mixture contained 1 U of AmpliTaq Gold DNA Polymerase (Applied Biosystems, Foster City, CA), 10 mM Tris–HCl, 50 mM KCl, 2.5 mM $MgCl_2$, 0.2 mM each deoxynucleoside triphosphate (dNTP), 0.2 μM batra primers (Valentini et al., 2016), 4 μM human blocking primer for the batra primers (to reduce human DNA amplification, 5′-TCACCCTCCTCAAGTATACTTCAAAGGCA-SPC3-3′; Valentini et al., 2016) and 0.2 μg/μL bovine serum albumin (Roche Diagnostic, Basel, Switzerland). The batra primers were 5′-labeled with an eight-nucleotide tag unique to each PCR replicate (with at least three differences between any pair of tags), allowing the assignment of each sequence to the corresponding sample during sequence analysis. The tags for the forward and reverse primers were identical for each PCR replicate. The PCR mixture was denatured at 95°C for 10 min, followed by 50 cycles of 30 s at 95°C, 30 s at 55°C and 1 min at 72°C, and a final elongation step at 72°C for 7 min. This step was performed in a room dedicated to amplified DNA with negative air pressure and physical separation from the DNA extraction rooms (with positive air pressure). Twelve replicate PCRs were run per filtration.

After amplification, the samples were titrated using capillary electrophoresis (QIAxcel; Qiagen GmbH) and purified using the MinElute PCR purification kit (Qiagen GmbH). Before sequencing, purified DNA was titrated again using capillary electrophoresis. The purified PCR products were pooled in equal volumes to achieve a theoretical sequencing depth of 300,000 reads per sample. PCR purification were performed in a room dedicated to amplified DNA analysis with negative air pressure and physically separated from the eDNA extraction room. All benches were decontaminated with 10% commercial bleach before and after each manipulation. Libraries preparations and sequencing were performed at Fasteris (Geneva, Switzerland). One and three libraries were prepared for the 2017 and 2018 surveys, respectively, using the MetaFast protocol (Fasteris, https://www.fasteris.com/dna/?q=content/metafast-protocol-amplicon-metagenomic-analysis), and paired-end sequencing (2×125 base pairs [bp]) was carried out on a Illumina Miseq sequencer (Illumina, San Diego, CA) with different MiSeq Flow Cell V3 for each library (Illumina, San Diego, CA) following the manufacturer's instructions. Four negative extraction controls and three negative PCR controls (ultrapure water) were amplified (12 replicates) and sequenced in parallel to sample processing to monitor possible contaminants.

Sequence reads were analyzed using programs implemented in the OBITools package (http://metabarcoding.org/obitools) (Boyer et al., 2016) following the protocol by Valentini et al. (2016). The forward and reverse reads were assembled using the *illuminapairedend* module with a minimum score of 40 and retrieving only joined sequence. The reads were then assigned to each sample (*ngsfilter* module). A separate dataset was created for each sample by splitting the original dataset in several files (*obisplit* module). After this step, each sample was analyzed individually before merging the taxon list for the final ecological analysis. Strictly identical sequences were clustered together (*obiuniq* module) and those shorter than 20 bp or with less than 10 of coverage

were excluded (*obigrep* module). The *obiclean* module was then run within a PCR product, to discard all sequences that result from PCR substitutions and indel errors. Taxonomic assignment of the molecular operational taxonomic units (MOTUs) was performed using module *ecotag* with the reference database of amphibians used in Valentini et al. (2016), in addition to one local reference database constructed for this study. MOTUs showing less than 98% similarity to the local reference database were removed. Finally, sequences below 0.003 of occurrence frequency per taxon and per library were discarded to account for bad sequence identification due to tag-jumps (Schnell, Bohmann, & Gilbert, 2015). These thresholds were empirically determined to clear all reads from the negative controls included in our global data production procedure as suggested in De Barba et al. (2014).

The 2017 library yielded 1,374,062 reads, of which 750,937 were retained after filtering, and assigned to 10 amphibian taxa. For the 2018 libraries, we obtained 8,568,468 reads of which 2,851,098 were retained, and assigned to 12 amphibians taxa. No reads were found in the extraction and PCR controls.

2.2 | Sampling of wild-caught newts

We visited 16 of the above sites in May–June 2018 to collect newts by dipnetting or with aquatic funnel traps. Sites were selected to cover the putative core of the invaded area, as well as nearby areas, which have high densities of amphibian breeding sites inhabited by crested newts. Buccal cells were sampled using cotton swabs, and DNA was extracted by the BioSprint Robotic workstation (Qiagen). A total of 126 newts were captured in 12 of these sites, and 116 of them were included in the genetic analyses. We analyzed additional samples from both species as references, that is, from Bern (BE, central Switzerland, $n = 10$ *T. cristatus*), Vaud (VD, western Switzerland, $n = 10$ *T. cristatus*), Ticino (TI, southern Switzerland, $n = 4$ *T. carnifex*), Geneva (GE, southwestern Switzerland, $n = 4$ *T. carnifex*), and Tuscany (TUS, western Italy, $n = 7$ *T. carnifex*). See details in File S1.

2.3 | Mitotyping and genotyping of wild-caught newts

We sequenced 112 samples from the Basel area and 14 reference samples (File S1) with the mitochondrial DNA (mtDNA) control region (CR), which features haplotypes discriminating *T. carnifex* and *T. cristatus*, using primers L-Uro and H-tRNAPhe-Uro (Dufresnes et al., 2016). Amplification were carried out in 25 μL reaction volumes, including 1 μL of each primers (10 μM), 7.5 μL of Qiagen Multiplex Primer Mix (MPMM, a premix including hot-start polymerase, dNTP, and buffer), 12.5 μL of milli-Q water and 3 μL of template DNA. PCRs ran as follows: 95°C for 15 min, 35 cycles of 94°C for 30 s, 53°C for 45 s and 72°C for 1 min, followed by 30 min at 60°C. Amplicons were verified on an agarose gel and Sanger-sequenced. Sequence chromatograms were visualized using MEGA and aligned by the muscle algorithm.

To assess their origin based on the detailed mitochondrial phylogeography by Canestrelli, Salvi, Maura, Bologna, and Nascetti (2012), all samples from the Basel area bearing a *T. carnifex* CR haplotype were also sequenced for the mtDNA ND4 gene, using a *T. carnifex*-specific primer pair (ND4 and LEU; Canestrelli et al., 2012; same protocol as CR). We further sequenced this fragment in seven *T. carnifex* reference samples (File S1).

To infer the nuclear nature of crested newts in the Basel area, we genotyped nine microsatellite loci previously used in invasion genetics on this system (Dufresnes et al., 2016 and references therein): Tcri13, Tcri29, Tcri35, Tcri36, A126, A8, D1, D5, and D127. Multiplex PCRs included 3 μL of template DNA, equal volumes of forward and reverse primer (10 ng/μL, as follows), completed with milli-Q water for a final reaction volume of 10 μL. Mix 1: Tcri13 (0.1 μL), Tcri35 (0.2 μL), and Tcri46 (0.1 μL); Mix 2 updated: Tcri29 (0.2 μL), Tcri36 (0.3 μL), and A126 (0.2 μL); Mix 4 updated: D1 (0.2 μL), D5 (0.2 μL), and A7 (0.2 μL); and Mix 5: A8 (0.15 μL) and D127 (0.2 μL). Amplicons were diluted with 30, 50, 20, and 30 μL of milli-Q water, respectively, and run on an ABI Prism 3100 genetic analyzer. Peaks were scored with GeneMapper 4.0 (Applied Biosystems). A total of 146 individual genotypes could be generated, including the 116 from the Basel area, 16 from reference *T. cristatus* and 14 from reference *T. carnifex* (File S1).

2.4 | Population genetics analyses of wild-caught newts

CR haplotypes (741 bp aligned) were matched against those of a previous study (637 bp; Dufresnes et al., 2016) to infer the species mitotype. ND4 haplotypes were matched against ND2 + ND4 concatenated haplotypes from Canestrelli et al. (2012), which provided detailed haplotype occurrence. For visualization, we performed a maximum-likelihood inference of collapsed ND4 haplotypes (636 bp) with PhyML (Guidon et al., 2010), using *T. cristatus* as an outgroup.

We analyzed the nuclear data in several ways. First, microsatellite genotypes were assigned to genetic groups by the Bayesian clustering algorithm of STRUCTURE (Pritchard, Stephens, & Donnelly, 2000). We ran 10 replicates for K = 1 to 11, each consisting of 110,000 iterations, including 10,000 of burn-in. Second, we summarized genetic variation by a principal component analysis (PCA) on individual allele frequency using the R package *adegenet*.

Third, we computed pairwise genetic distances (F_{st}) for populations with $n \geq 5$ with *hierfstat.* To characterize putative hybrid individuals, we also ran NewHybrids (Anderson & Thompson, 2002) for 200,000 iterations following a burn-in of 20,000, using our reference genotypes as parental individuals. This program assigns individuals to hybrid classes, in our case parental, F1, F2, and first-generation backcrosses in either direction.

3 | RESULTS

3.1 | eDNA metabarcoding survey

Preliminary eDNA analyses of five sites in 2017 detected *T. carnifex* at BL3 and BL191 and *T. cristatus* at BL173 and BL191 (Table 1). Our 2018 analyses only confirmed the presence of *T. carnifex* at the latter, and also detected it in two newly surveyed ponds from the same network (BL196 and BL198). Outside this area, only the native *T. cristatus* was detected across 12 other sites, delineating the putative introduction range to less than 4 km^2 (Figure 1, Table 1). Detection was weak (only one replicate, with <100 reads) and below the threshold established by SPYGEN (following De Barba et al., 2014; see section 2.1) for two sites where no crested newt was captured (Figure 1, Table 1, File S1). At site BL195, eDNA did not detect *Triturus*, while we did capture one larva (File S1). At least 10 other amphibians were also detected throughout the region; full details can be found in File S1, including number of sequence reads and replicate detections for every species per site.

3.2 | Mitochondrial variation in wild-caught newts

Mitochondrial genetics of wild-caught adults yielded similar results as eDNA. All newts captured within the identified

TABLE 1 Newt composition of 22 sample sites surveyed by environmental DNA (eDNA) (2018 + 2017), field capture (2018; *n*: number of newts caught), and multilocus population genetics

Site	eDNA monitoring	eDNA detection	*n*	mtDNA nature	Nuclear nature
Introduction area					
BL191	2018/2017	*Triturus carnifex*/*T. carnifex-cristatus*	10	10 *T. carnifex*	10 pure *T. carnifex*
BL196	2018	*T. carnifex*	7	7 *T. carnifex*	7 pure *T. carnifex*
BL198	2018	*T. carnifex*	10	10 *T. carnifex*	10 pure *T. carnifex*
BL899	2018	None	1	1 *T. carnifex*	1 pure *T. carnifex*
BL3G	2018/2017	None/*T. carnifex*	1	1 *T. carnifex*	1 pure *T. carnifex*
BL3S	2018	None	–	–	–
Surrounding areas					
BL173U	2018/2017	*T. cristatus*/*T. cristatus*	10	10 *T. cristatus*	6 pure *cristatus* + 4 backcrosses
BL173O	2018	*T. cristatus*	12	11 *T. cristatus*	10 pure *cristatus*
BL17	2018	*T. cristatus*	16	12 *T. cristatus*	15 pure *cristatus*
BL171	2018	*T. cristatus*	14	14 *T. cristatus*	14 pure *cristatus*
BL195	2018	None	1	–	–
BL45	2018	*T. cristatus*	–	–	–
BL51	2018	None	–	–	–
BL53	2018	None	–	–	–
BL601	2018	*T. cristatus*[a]	0	–	–
BL618	2018	*T. cristatus*	20	15 *T. cristatus*	15 pure *cristatus*
BL62	2017	*T. cristatus*	–	–	–
BL620	2018	*T. cristatus*	0	–	–
BL623	2017	None	–	–	–
BL627	2018	*T. cristatus*[a]	0	–	–
BS10	2018	*T. cristatus*	7	6 *T. cristatus*	6 pure *cristatus*
BS4	2018	*T. cristatus*	18	15 *T. cristatus*	15 pure *T. cristatus*

Abbreviation: mtDNA, mitochondrial DNA.

[a]Below thresholds established by SPYGEN (see section 2.1). Hyphens: not surveyed/analyzed.

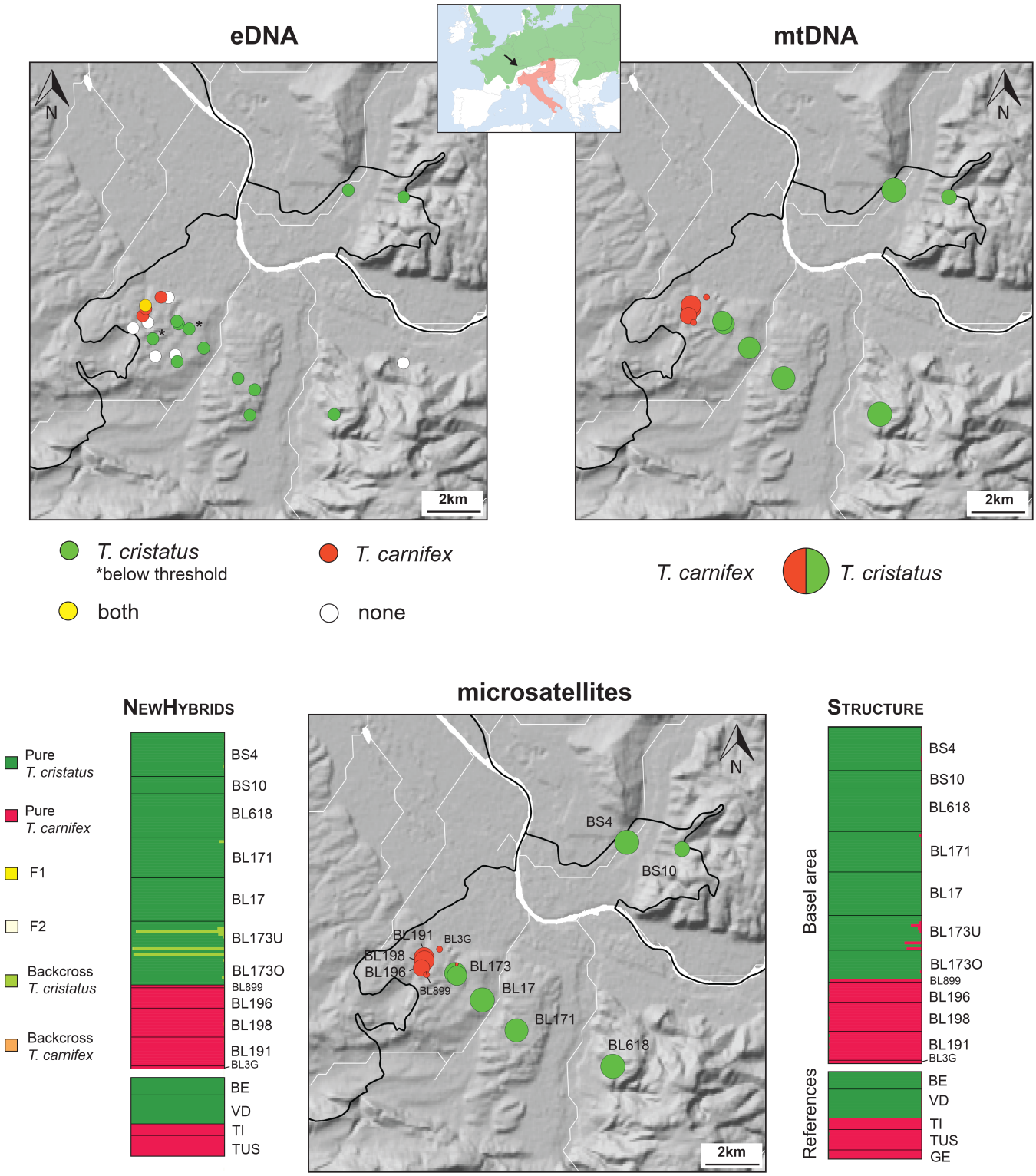

FIGURE 1 Comparative assessment of the distribution of *Triturus cristatus* (native) and *Triturus carnifex* (invasive) in the area of Basel, northwestern Switzerland, from water samples (environmental DNA [eDNA], 2017–2018 combined) and wild-caught adults (mitochondrial DNA [mtDNA] and nuclear microsatellites). Circle sizes are proportional to sample sizes. The small insert map on top shows the natural distribution of the two species (red: *T. carnifex*; green: *T. cristatus*) as taken from the IUCN Red List database (https://www.iucnredlist.org/), and the location of the study area (arrow). Bar plots show individual assignment probabilities for each nuclear clusters (STRUCTURE) and hybrid classes (NewHybrids)

area of introduction featured *T. carnifex* CR haplotypes (Figure 1, Table 1, File S1). We also found a single newt (with *T. carnifex* mtDNA) at a fifth site (BL899). Specimens from all other populations possessed *T. cristatus* mtDNA (Figure 1).

Analyses of the hypervariable mitochondrial ND4 gene show that the introduced *T. carnifex* belong to the Central Italian clade of this taxon (clade C in Canestrelli et al., 2012). The two collapsed haplotypes CIII10/11 and CII3/4/7 (Canestrelli et al., 2012) naturally occur throughout the southern Po Plain and the foothills of the Northern Apennine Mountains (Figure 2). In contrast, newts that invaded the Geneva Basin in southwestern Switzerland belong to the southern-Italian clade S (ND4 collapsed haplotypes SI2, SI4, SI5, SI1/3/19/31). Other Swiss *T. carnifex* naturally present in Ticino bear Central-Italian, yet different ND4 haplotypes: CI2/CII9, and a previously unsampled one, C-TI.

3.3 | Population genetics of wild-caught newts

Analyses of microsatellite genotypes recovered the two gene pools of *T. carnifex* and *T. cristatus* as two STRUCTURE groups (Figure 1, File S2), two well-defined clusters along the first component of the PCA (File S3), and two sets of populations featuring strong pairwise genetic distances (File S4). Their geographic distribution fully matches mtDNA: There was not a single case of cyto-nuclear discordance, and the *T. carnifex* nuclear alleles are restricted to the putative introduction range. A few individuals from the closest *T. cristatus* populations (BL173) however featured admixture coefficients consistent with genetic introgression (Figure 1). Assignment to hybrid classes by NewHybrids identified three of them as first-generation backcrosses with *T. cristatus*, while the rest of our samples had the highest probabilities to belong to the parental classes.

Nuclear variation within *T. carnifex* confirmed the central-Italian origin of Basel invaders. The second PCA axis (File S3) and pairwise genetic distances (File S4) distinguish them from the newts of the south-Italian clade (Tuscany and Geneva), and group them close (but without overlapping) to our reference samples from southern Switzerland (Ticino).

4 | DISCUSSION

4.1 | Emerging invasion of Italian crested newts

The metabarcoding eDNA approach applied to water samples discriminated the crested newts *T. cristatus* and *T. carnifex* and characterized a new introduction of the latter outside its natural range, namely the region of Basel in northwestern Switzerland. Population genetics of wild-caught adults confirmed eDNA results and provided additional insights into two interesting aspects of the introduction.

First, the animals all originated from the Po Plain in Italy, and were not translocated from the Geneva invasive range in Western Switzerland (Arntzen & Thorpe, 1999), where newts belong to a different Italian lineage (Dufresnes et al., 2016). The Basel introduction likely stems from intentional or accidental releases from a pet store formerly located

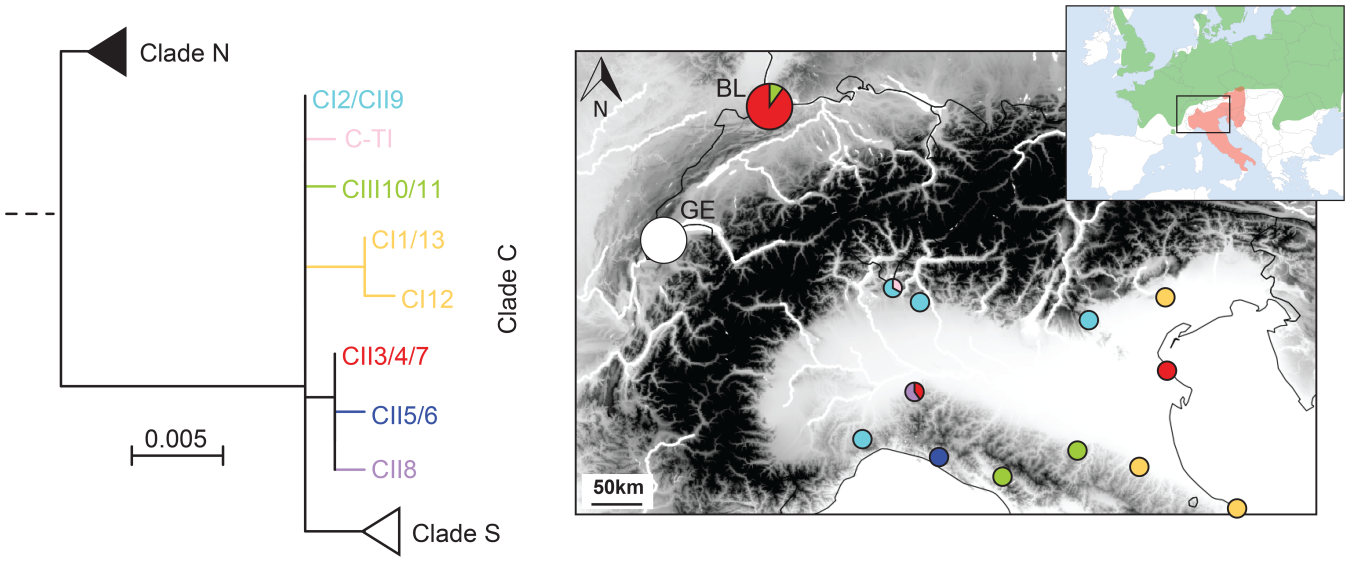

FIGURE 2 Phylogenetic relationships between ND4 *Triturus carnifex* haplogroups and North-Italian haplotypes (clade C). Labels indicate collapsed ND2 + ND4 haplotypes from Canestrelli et al. (2012). Clade N is restricted to Slovenia and Austria, whereas clade C is distributed south of the Apennine Mountains. *Triturus cristatus* was used as outgroup

nearby, or hobbyists/uninformed naturalists (Dubey et al., 2019). Second, we documented limited genetic admixture with the native *T. cristatus* —restricted at site BL173U, less than 2 km away from the introduction area— and no cyto-nuclear disequilibrium. *Triturus* newts commonly hybridize in natural (Arntzen, Wielstra, & Wallis, 2014; Maletzky et al., 2008) and invasive ranges, which contributes to the spread of *T. carnifex* genes (Brede, 2015; Dufresnes et al., 2016; Meilink, Arntzen, van Delft, & Wielstra, 2015; Wielstra et al., 2016). Widespread mtDNA introgression has been reported in *Triturus* invasions (Dufresnes et al., 2016), as also seen from their moving natural hybrid zones (e.g., Wielstra et al., 2017). Here, *T. cristatus* probably occurred in low densities (and were historically absent; Labhardt & Schneider, 1981) and were thus rapidly replaced at infested sites (detected only in 2017 in BL191, but not in 2018) without leaving genetic traces.

Based on the initial photographic suspicion (2012), and the detection of backcrosses (i.e., second-generation hybrids), the first releases of *T. carnifex* are at least 7 years old. The invaded area yet remains relatively narrow, probably due to the low dispersal rates of crested newts (Cayuela, Schmidt, Weinbach, Besnard, & Joly, 2019), even within single pond clusters in our study area (e.g., between BL173U and BL173O; Schwizer, 2007). Retrospectively, it supports that the wider and scattered geographic distribution of *T. carnifex* in other invasive ranges were likely promoted by human translocations (Dufresnes et al., 2016).

Although presently localized, this emerging invasion calls for an immediate management response (eradication) to prevent further spread, introgression and replacement of the native species. Amphibian and reptile taxa from the Apennine Peninsula, such as *T. carnifex*, show a remarkable ability to acclimatize to environmental conditions north of the Alps, resulting in uncontainable expansions and costly biological (i.e., declines of local species) and economic consequences (eradication efforts; Dubey et al., 2019).

4.2 | eDNA for the surveillance of aquatic invasive species

The Basel *T. carnifex* invasion offers an opportunity to appreciate the potential of eDNA metabarcoding for monitoring cryptic emerging invasions. To the best of our knowledge, this is the first comparative framework combining eDNA with direct multilocus genotyping of live-caught specimens in an invasion context. Overall, results from both approaches closely matched, validating the eDNA metabarcoding technology as a powerful tool to unravel and map cryptic invaders.

We outline several major strengths for the use of eDNA in an invasion context. First, the closely related *T. cristatus* and *T. carnifex* were reliably differentiated despite that our reference database was restricted to sequences from another *T. carnifex* clade (clade S introduced in the Geneva Basin; Dufresnes et al., 2016), deeply diverged from the Basel invaders (clade C). Note also that the technology discriminated and detected another notorious group of aliens: the marsh frogs (*Pelophylax ridibundus* sensu lato), which are invading the Swiss plateau (Dufresnes et al., 2018). Second, populations from both the native and invasive species could be efficiently mapped, as expected given the high detection probability of eDNA (up to 0.97) compared to traditional field surveys (Smart, Tingley, Weeks, van Rooyen, & McCarthy, 2015; Valentini et al., 2016). Third, the average costs per site (fieldwork + analyses) were ~60% lower for eDNA metabarcoding (~600€) than population genetics (~1,470€). Cost-effectiveness is one major advantage of eDNA to optimize the use of conservation budgets, especially for reliable species detection (Dejean et al., 2012; see also Smart et al., 2016). Hence, eDNA metabarcoding appears adequate for affordable, early detection and reliable range mapping of introduced and invasive species, without the administrative and ethical challenges of capturing live animals.

There are nevertheless several potential limitations to point out. First, regional range inferences of native versus exotic species might be skewed in case of asymmetric hybridization, leading to cyto-nuclear discordance and thus misidentification from mtDNA-based methods. This should, however, only concern old invasions where the local species had already been genetically replaced through massive gene flow (Dufresnes et al., 2016). Some hybrids, especially if in low density and backcrossed, could also remain unnoticed (e.g., BL173). Second, recently-diverged taxa still remain undistinguishable by current metabarcoding methods, especially in some complex taxonomic groups (e.g., water frogs, *Pelophylax* sp., where interspecies mtDNA transfer is possible; Spolsky & Uzzell, 1984), which then requires targeted assessments (e.g., Dufresnes et al., 2017). However, this could be rapidly improved by sequencing additional mtDNA fragments and complementing reference databases, or alternatively through species-specific detections. Finally, eDNA did not systematically detect newts in all sites, for example, BL3, BL195, BL899, where only a single individual was captured in each (Table 1). eDNA detection is density-dependent and may thus overlook small populations (Schmidt et al., 2013).

Surveying large areas for invasive species is notoriously challenging because of the amount of required resources (money, personnel). eDNA may be a relatively cheap solution (Deiner et al., 2017; Ficetola, Miaud, Pompanon, & Taberlet,

2008) to detect new invaders at an early stage when eradication is still possible (Jerde, Mahon, Chadderton, & Lodge, 2011). Our study contributes to this growing body of evidence, by confirming that eDNA metabarcoding results are consistent with conventional invasion genetics, and extends this approach to cryptic invasions where genetic pollution is a key threatening process. When hybridization may occur, eDNA surveys can be used to design targeted monitoring. The combination of these methods holds great promise for multiscale assessments necessary to identify, understand, and ultimately manage biological invasions.

ACKNOWLEDGMENTS

We thank S. Ursenbacher for advice, C. Stickelberger and T. Reissner for collecting animals in the field, as well as SPYGEN staff for eDNA labwork. We are also grateful for the useful comments of two anonymous reviewers, and the Associate Editor Liba Goldstein. This study was funded by the Federal Office for the Environment (FOEN/OFEV/BAFU; Francis Cordillot) and the Abteilung Naturförderung of canton Bern.

CONFLICT OF INTEREST

We declare the following conflict of interest: T.D. cofounded SPYGEN, the company that developed the eDNA metabarcoding approach used in this study.

AUTHOR CONTRIBUTIONS

S.D. designed the study. S.Z., B.R.S., and P.R. conducted fieldwork. T.D. performed the eDNA analyses. L.F. provided reference samples. C.D. conducted the population genetic analyses and drafted the manuscript, which was improved by all coauthors.

DATA ACCESSIBILITY

Mitochondrial sequences are available on GenBank (MK415776-MK415792); the eDNA results are available in File S1a; mtDNA haplotype and microsatellite genotypes are accessible in File S1c.

ETHICS STATEMENT

Newts were captured and sampled for DNA using noninvasive buccal swabs, under permits issued by the section Natur und Landschaft, Landwirtschaftliches Zentrum Ebenrain, canton Basel-Landschaft and section Natur Landschaft Bäume, Stadtgärtnerei, canton Basel-Stadt. All animal were subsequently released unharmed.

ORCID

Christophe Dufresnes https://orcid.org/0000-0002-8497-8908
Benedikt R. Schmidt https://orcid.org/0000-0002-4023-1001
Luca Fumagalli https://orcid.org/0000-0002-6648-2570

REFERENCES

Anderson, E. C., & Thompson, E. A. (2002). A model-based method for identifying species hybrids using multilocus genetic data. *Genetics*, *160*, 1217–1229.

Arntzen, J. W., & Thorpe, R. S. (1999). Italian crested newts (*Triturus carnifex*) in the Basin of Geneva: Distribution and genetic interactions with autochthonous species. *Herpetologica*, *55*, 423–433.

Arntzen, J. W., Wielstra, B., & Wallis, G. P. (2014). The modality of nine *Triturus* newt hybrid zones, assessed with nuclear, mitochondrial and morphological data. *Biological Journal of the Linnean Society*, *113*, 604–622.

Biggs, J., Ewald, N., Valentini, A., Gaboriaud, C., Dejean, T., Griffiths, R., … Dunn, F. (2015). Using eDNA to develop a national citizen science-based monitoring programme for the great crested newt (*Triturus cristatus*). *Biological Conservation*, *183*, 19–28.

Boyer, F., Mercier, C., Bonin, A., Le Bras, Y., Taberlet, P., & Coissac, E. (2016). OBITOOLS: A UNIX-inspired software package for DNA metabarcoding. *Molecular Ecology Resources*, *16*, 176–182.

Brede, E. (2015). Beam Brook revisited: A molecular study of a historically introduced non-native amphibian (*Triturus carnifex*) and its limited introgression into native UK *Triturus cristatus* populations. *Amphibia-Reptilia*, *36*, 287–299.

Canestrelli, D., Salvi, D., Maura, M., Bologna, M. A., & Nascetti, G. (2012). One species, three Pleistocene evolutionary histories: Phylogeography of the Italian crested newt, *Triturus carnifex*. *PLoS One*, *7*, e41754.

Cayuela, H., Schmidt, B. R., Weinbach, A., Besnard, A., & Joly, P. (2019). Multiple density-dependent processes shape the dynamics of a spatially structured amphibian population. *Journal of Animal Ecology*, *88*, 164–177.

Clusa, L., Miralles, L., Basanta, A., Escot, C., & Garcia-Vazquez, E. (2017). eDNA for detection of five highly invasive molluscs. A case study in urban rivers from the Iberian Peninsula. *PLoS ONE*, *2*, e0188126.

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.

De Barba, M., Miquel, C., Boyer, F., Mercier, C., Rioux, D., Coissac, E., & Taberlet, P. (2014). DNA metabarcoding multiplexing and validation of data accuracy for diet assessment: Application to omnivorous diet. *Molecular Ecology Resources*, *14*, 306–323.

Deiner, K., Bik, H. M., Mächler, E., Seymour, M., Lacoursière-Roussel, A., Altermatt, F., … Bernatchez, L. (2017). Environmental DNA metabarcoding: Transforming how we survey animal and plant communities. *Molecular Ecology*, *26*, 5872–5895.

Dejean, T., Valentini, A., Miquel, C., Taberlet, P., Bellemain, E., & Miaud, C. (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*, 953–959.

Doherty, T. S., Glen, A. S., Nimmo, D. G., Ritchie, E. G., & Dickman, C. R. (2016). Invasive predators and global biodiversity loss. *Proceedings of the National Academy of Sciences of the United States of America*, *113*, 11261–11265.

Dubey, S., Lavanchy, G., Thiébaud, J., & Dufresnes, C. (2019). Herps without borders: A new newt case and a review of transalpine alien introduction in Western Europe. *Amphibia-Reptilia*, *40*, 13–27.

Dufresnes, C. (2019). *Amphibians of Europe, North-Africa and the Middle East*. London, England: Bloomsbury.

Dufresnes, C., Di Santo, L., Leuenberger, J., Schuerch, J., Mazepa, G., Grandjean, N., … Dubey, S. (2017). Cryptic invasion of Italian pool frogs (*Pelophylax bergeri*) across Western Europe unraveled by multilocus phylogeography. *Biological Invasions*, *19*, 1407–1420.

Dufresnes, C., Leuenberger, J., Amrhein, V., Bühler, C., Thiébaud, J., Bojnenstengel, T., & Dubey, S. (2018). Invasion genetics of marsh frogs (*Pelophylax ridibundus* sensu lato) in Switzerland. *Biological Journal of the Linnean Society*, *123*, 402–410.

Dufresnes, C., Pellet, J., Bettinelli-Riccardi, S., Thiébaud, J., Perrin, N., & Fumagalli, L. (2016). Massive genetic introgression in threatened northern crested newts (*Triturus cristatus*) by an invasive congener (*T. carnifex*) in western Switzerland. *Conservation Genetics*, *17*, 839–846.

Fahrbach, M., & Gerlach, U. (2018). *The genus Triturus*. Frankfurt, Germany: Chimaira Buchhandelsgesellschaft mbH.

Ficetola, G. F., Miaud, C., Pompanon, F., & Taberlet, P. (2008). Species detection using environmental DNA from water samples. *Biology Letters*, *4*, 423–425.

Fukumoto, S., Ushimaru, A., & Minamoto, T. (2015). A basin-scale application of environmental DNA assessment for rare endemic species and closely related exotic species in rivers: A case study of giant salamanders in Japan. *Journal of Applied Ecology*, *52*, 358–365.

Gantz, C. A., Renshaw, M. A., Erickson, D. M., Lodge, D. M., & Egan, S. P. (2018). Environmental DNA detection of aquatic invasive plants in lab mesocosm and natural field conditions. *Biological Invasions*, *20*, 1–18.

Guidon, S., Dufayard, J. F., Lefort, V., Anisimova, M., Hordijk, W., & Gascuel, O. (2010). New algorithms and methods to estimate maximum-likelihood phylogenies: Assessing the performance of PhyML 3.0. *Systematic Biology*, *59*, 307–321.

Hulme, P. E., Bacher, S., Kenis, M., Klotz, S., Kühn, I., Minchin, D., … Vilà, M. (2008). Grasping at the routes of biological invasions: A framework for integrating pathways into policy. *Journal of Applied Ecology*, *45*, 403–414.

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.

Keller, R. P., Lodge, D. M., & Finnoff, D. C. (2007). Risk assessment for invasive species produces net bioeconomic benefits. *Proceedings of the National Academy of Sciences*, *104*, 203–207.

Labhardt, F., & Schneider, C. (1981). Überblick über die Amphibienbestände in den Kantonen Basel-Landschaft und Basel-Stadt. Tätigkeitsber. *Naturforschende Gesellschaft Baselland*, *31*, 185–223.

Lahoz-Montfort, J. J., Guillera-Arroita, G., & Tingley, R. (2016). Statistical approaches to account for false-positive errors in environmental DNA samples. *Molecular Ecology Resources*, *16*, 673–685.

Lowry, E., Rollinson, E. J., Laybourn, A. J., Scott, T. E., Aiello-Lammens, M. E., Gray, S. M., … Gurevitch, J. (2013). Biological invasions: A field synopsis, systematic review and database of the literature. *Ecology and Evolution*, *3*, 182–196.

Mahon, A. R., & Jerde, C. L. (2016). Using environmental DNA for invasive species surveillance and monitoring. *Methods in Molecular Biology*, *1452*, 131–142.

Maletzky, A., Mikulicek, P., Franzen, M., Goldschmid, A., Gruber, H.-J., Horak, A., & Kyek, M. (2008). Hybridization and introgression between two species of crested newts (*Triturus cristatus* and *T. carnifex*) along contact zones in Germany and Austria: Morphological and molecular data. *Herpetological Journal*, *18*, 1–15.

Meilink, W. R. M., Arntzen, J. W., van Delft, J. J. C. W., & Wielstra, B. (2015). Genetic pollution of a threatened native crested newt species through hybridization with an invasive congener in The Netherlands. *Biological Conservation*, *184*, 145–153.

Nevers, M. B., Byappanahalli, M. N., Morris, C. C., Shively, D., Przybyla-Kelly, K., Spoljaric, A. M., … Roseman, E. F. (2018). Environmental DNA (eDNA): A tool for quantifying the abundant but elusive round goby (*Neogobius melanostomus*). *PLoS One*, *13*, 0191720.

Pimentel, D., Zuniga, R., & Morrison, D. (2005). Update on the environmental and economic costs associated with alien-invasive species in the United States. *Ecological Economics*, *52*, 273–288.

Pont, D., Rocle, M., Valentini, A., Civade, R., Jean, P., Maire, A., … Dejean, T. (2018). Environmental DNA reveals quantitative patterns of fish biodiversity in large rivers despite its downstream transportation. *Scientific Reports*, *8*, 10361.

Pritchard, J. K., Stephens, M., & Donnelly, P. (2000). Inference of population structure using multilocus genotype data. *Genetics*, *155*, 945–959.

Pysek, P., & Richardson, D. M. (2010). Invasive species, environmental change and management, and health. *Annual Review of Environment and Resources*, *35*, 25–55.

Schmidt, B. R., Kéry, M., Ursenbacher, S., Hyman, O. J., & Collins, J. P. (2013). Site occupancy models in the analysis of environmental DNA presence/absence surveys: A case study of an emerging amphibian pathogen. *Methods in Ecology and Evolution*, *4*, 646–653.

Schneider, J., Valentini, A., Dejean, T., Montarsi, F., Taberlet, P., Glaizot, O., & Fumagalli, L. (2016). Detection of invasive mosquito vectors using environmental DNA (eDNA) from water samples. *PLoS One*, *11*, e0162493.

Schnell, I. B., Bohmann, K., & Gilbert, M. T. (2015). Tag jumps illuminated—Reducing sequence-to-sample misidentifications in metabarcoding studies. *Molecular Ecology Resources*, *15*, 1289–1303.

Schwizer, T. (2007). *Population dynamics of a subdivided population of the crested newt (Triturus cristatus)* (MSc thesis). University of Zurich, Switzerland.

Secretariat of the Convention on Biological Diversity. (2006). *Global biodiversity outlook 2*. Montreal, Canada: Author.

Smart, A. S., Tingley, R., Weeks, A. R., van Rooyen, A. R., & McCarthy, M. A. (2015). Environmental DNA sampling is more sensitive than a traditional survey technique for detecting an aquatic invader. *Ecological Applications*, *25*, 1944–1952.

Smart, A. S., Weeks, A. R., van Rooyen, A. R., Moore, A., McCarthy, M. A., & Tingley, R. (2016). Assessing the cost-efficiency of environmental DNA sampling. *Methods in Ecology and Evolution*, *7*, 1291–1298.

Spolsky, C., & Uzzell, T. (1984). Natural interspecies transfer of mitochondrial DNA in amphibians. *Proceedings of the National Academy of Sciences*, *81*, 5802–5805.

Taberlet, P., Bonin, A., Zinger, L., & Coissac, E. (2018). *Environmental DNA*. Oxford, England: Oxford University Press.

Tréguier, A., Paillisson, J.-M., Dejean, T., Valentini, A., Schlaepfer, M. A., & Roussel, J.-M. (2014). Environmental DNA surveillance for invertebrate species: Advantages and technical limitations to detect invasive crayfish *Procambarus clarkia* in freshwater ponds. *Journal of Applied Ecology*, *51*, 871–879.

Valentini, A., Taberlet, P., Miaud, C., Civade, R., Herder, J., Thomsen, P. F., & Dejean, T. (2016). Next-generation monitoring of aquatic biodiversity using environmental DNA metabarcoding. *Molecular Ecology*, *25*, 929–942.

Wielstra, B., Burke, T., Butlin, R. K., Avci, A., Uzum, N., Bozkurt, E., … Arntzen, J. W. (2017). A genomic footprint of hybrid zone movement in crested newts. *Evolution Letters*, *1*, 93–101.

Wielstra, B., Burke, T., Butlin, R. K., Schaap, O., Shaffer, H. B., Vrieling, K., & Arntzen, J. W. (2016). Efficient screening for "genetic pollution" in an anthropogenic crested newt hybrid zone. *Conservation Genetics Resources*, *8*, 553–560.

SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of this article.

How to cite this article: Dufresnes C, Déjean T, Zumbach S, et al. Early detection and spatial monitoring of an emerging biological invasion by population genetics and environmental DNA metabarcoding. *Conservation Science and Practice*. 2019;1:e86. https://doi.org/10.1111/csp2.86