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ORIGINAL ARTICLE

Detection of environmental DNA from amphibians in Northern Europe applied in citizen science

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Abstract

Many species of amphibians in Northern Europe are threatened and the local distributions are rarely described in detail. Application of modern molecular methods provides an important supplementary tool for monitoring the distribution and diversity of amphibians. For this purpose, we designed, tested, validated, and optimized 14 species-specific assays on genomic DNA extracted from tissue samples to use for quantitative polymerase chain reaction (qPCR) setups targeting mitochondrial DNA from amphibians in freshwater samples. The tests confirmed species specificity for all assays. Considering a systematic definition of the limit of detection for each of the assays, the presented qPCR assays are unlikely to return false positive detection from any co-occurring species in northern Europe. For field validation, the qPCR assays were applied in a large-scale nationwide citizen science project in which sampling and qPCR analysis was carried out by high school students. Data from the citizen science project returned the expected results when compared to the known regional distribution of the target species and confirmed the presence of nine out of 14 Danish species of amphibians in the collected freshwater samples. Four out of 2550 qPCR test sets carried out by the high school students required a professional reanalysis in multiple replicates due to initial unexpected results. This emphasizes that efforts from citizen science may generate large amounts of valuable data, as long as the results are carefully scrutinized by experts.

KEYWORDS
Amphibia, Anura, eDNA, qPCR, quantitative PCR, real-time polymerase chain reaction

1 | INTRODUCTION

Amphibians are threatened worldwide (Houlahan et al., 2000; McCallum, 2007; Stuart et al., 2004) and high decline rates have been found in Europe due to an increase in industrialized agriculture (Cox et al., 2022) and landscape fragmentation (Araújo et al., 2006; Beebee & Griffiths, 2005; Fog et al., 2019), habitat loss and changes in water and soil quality (Bishop et al., 2012). To improve our

knowledge on amphibian decline and conservation efforts, better and more efficient tools for monitoring are needed (Ficetola et al., 2019; McKee et al., 2015; Pilliod et al., 2014). Conventional amphibian surveys require trained field biologists and include monitoring mating calls or looking for tadpoles. Observing amphibians is difficult as many species are nocturnal and live a hidden life, and the optimal period for monitoring is often weather dependent, and short as this is usually during the breeding season. This can make conventional monitoring complicated and costly. Analysis of environmental DNA (eDNA) in collected freshwater samples offers an attractive alternative monitoring approach (Ficetola et al., 2019; Thomsen et al., 2016; Thomsen, Kielgast, Iversen, Wiuf, et al., 2012; Valentini et al., 2016). Application of species-specific monitoring has demonstrated the ability to detect traces of eDNA in a broad array of environmental samples (Agersnap et al., 2017; Jensen et al., 2018; Székely et al., 2021). Several studies have used eDNA for amphibian monitoring, the majority focusing on a limited number of species such as *Pelophylax lessonae* (Eiler et al., 2018), *Triturus cristatus* (Biggs et al., 2015; Thomsen, Kielgast, Iversen, Wiuf, et al., 2012), *Rana catesbeiana* (Ficetola et al., 2008), *Ascaphus montanus* and *Dicamptodon aterrimus* (Goldberg et al., 2011), *Cryptobranchus a. alleganiensis* (Olson et al., 2012), *Ambystoma cingulatum*, *Ambystoma bishopi*, *Notophthalmus perstriatus*, and *Lithobates capito* (McKee et al., 2015). Monitoring efforts using eDNA have been shown to outperform traditional monitoring efforts regarding species detection (Dejean et al., 2011; Jo et al., 2020; Sigsgaard et al., 2015) and species richness (Sasso et al., 2017; Thomsen, Kielgast, Iversen, Møller, et al., 2012; Valentini et al., 2016). However, early reports on the increased detection of species using eDNA-based monitoring compared to conventional methods supplied no information on the limit of detection (LOD) for the assay used (Klymus et al., 2019).

Following the taxonomy listed by Speybroeck et al. (2010) and Dubois and Bour (2010), Denmark currently has 11 registered species of frogs and toads (Anura: *Bombina bombina*, *Bufo bufo*, *Bufo calamita*, *Bufo viridis*, *Hyla arborea*, *Pelobates fuscus*, *Pelophylax ridibundus*, *Pelophylax* kl. *esculentus*, *Rana arvalis*, *Rana dalmatina*, and *Rana temporaria*) and three species of newts (Urodela: *Ichthyosaura alpestris*, *Lissotriton vulgaris*, and *Triturus cristatus*) (Table 1). We here abstain from using the generic names: "Bufotes," "Epidalea," and "Pseudoepidalea," and instead use the genus name: "Bufo," as this taxonomy is in line with the recommendations by Speybroeck et al. (2010). The mitochondrial genome of *Pelophylax* kl. *esculentus*—a cross between *P. ridibundus* and *P. lessonae*—will be identical to one of the klepton's parent species (Hauswaldt et al., 2012). *Pelophylax lessonae* does not occur in Denmark, but is included in the study to ensure the ability to detect eDNA traces from *Pelophylax* kl. *esculentus*. *Pelophylax ridibundus* only occurs in Denmark on Bornholm (Fog et al., 1997) - a remote Danish island in the southern Baltic Sea (Hoffmann et al., 2015). Hence, any detection of eDNA from *P. lessonae* or *P. ridibundus* is considered to be a confirmation of the presence of *Pelophylax* kl. *esculentus*, resulting from the detection of eDNA from one of the parent mitochondrial DNA genomes.

The distribution of amphibians in Denmark is well described at a regional scale (Fog et al., 1997, 2019). However, at a local scale, information on distributions is often scarce. More knowledge on the detailed distribution of these species is important for future assessments of amphibian diversity in freshwater habitats. This is especially valuable during environmental impact assessments and when planning conservation activities targeting amphibians, and for urban constructions which often require detailed knowledge of amphibian distribution to avoid destruction of delicate habitats. Within the European Union, this is especially the case for the amphibian species covered by the species-protection in Habitat Directive; the so-called Annex IV species (Council Directive 92/43/EEC, 1992). Eight of the species monitored in this study are Annex IV species and includes: *Bombina bombina*, *Bufo calamita*, *Bufo viridis*, *H. arborea*, *P. fuscus*, *R. arvalis*, *R. dalmatina*, and *T. cristatus*.

To ensure adequate validation of the species-specific qPCR assays rigorous testing is required. This involves multiple validation steps (Langlois et al., 2020; Thalinger et al., 2021). The aim of this study was divided into two parts, where the first part was to have a professional researcher in charge of designing, optimizing, and validating species-specific qPCR assays in a uni-directional workflow laboratory that is dedicated for eDNA work. These assays are to target 14 species of amphibians in Northern Europe, that all are known to occur in Denmark. The second part involved collection of water samples by high school students in Denmark who volunteered to be a part of the citizen science project at the Natural History Museum of Denmark (NHMD) that is called "DNA and Life" (Danish: "DNA og Liv"), here abbreviated "DL" (Tøttrup et al., 2021), focusing mainly on amphibians, and at collecting and processing eDNA data to answer specific research questions on the distribution of fauna in freshwater and marine habitats. By including citizen science-collected samples our goal was to obtain a broader geographic coverage of the distribution of amphibians in Denmark, without investing too much time and money in fieldwork and allowing for sampling at remote locations. The students also partly carried out the final laboratory analysis in a second separate laboratory using the validated species-specific qPCR assays under professional supervision. The results from the qPCR analysis were compared with current knowledge on the regional distribution of the target species. Since we wanted to monitor many amphibians eDNA metabarcoding could have been an alternative approach, as comparison with genetic databases also could help monitor eDNA from amphibians in a single setup, but to allow for immediate interpretation of the results by the students we decided on using qPCR with multiple assays, as this is much more simple to analyze straight away. Analysis of huge datasets generated from metabarcoding of eDNA would have involved several complicated bioinformatic steps that would have been incomprehensible to the students.

It can be difficult to obtain a high number of samples across a broad geographic area. Time and money can quickly end up being the limiting factor determining the number of samples collected and brought back to the laboratory. A comparable citizen science approach has been carried out focusing on one species; *Triturus*

TABLE 1 Primer and probe qPCR detection systems for 14 amphibian species known from Northern Europe. Concentrations for primers and probes are optimal final concentrations in the qPCR reaction. Probes are 5′-end modified with a FAM-fluorescent dye and equipped with a black hole quencher-1 (BHQ1)-modification at the 3′-end. All primers and probes target a fragment in the mitochondrial genome. The primers and probe targeting *Pelophylax lessonae* were designed from sequences originating from *P. lessonae*, but were applied in qPCR test with the aim of finding eDNA from *Pelophylax* kl. *esculentus*, as *P.* kl. *esculentus* occurs in Denmark and *P. lessonae* does not (Fog et al., 1997, 2019). The molecular weight is given in Daltons (Da), and the target fragment length is in base pairs (bp). The sequence of the targeted fragment is listed in Table S1.

Order, family	Target species	Primer (F and R) and probe name (P)	Sequence, primer and probe 5′ → 3′ direction, with FAM and BHQ1 modifications	Optimal primer−/probe concentration per individual qPCR reaction (nM)	Molecular weight of dsDNA (Da)	Target fragment length (bp)
Anura, Bombinatoridae	*Bombina bombina*	Bombom_F15007	TGCAATCCTCCGATCAATTCCA	200		
	Bombina bombina	Bombom_R15086	GCAGGAGGGCAAGGATGAG	1000		
	Bombina bombina	Bombom_P15029	AATAAACTAGGAGGGGTACTAGCCC	450	49308.1	80
Anura, Bufonidae	*Bufo bufo*	Bufbuf_cytb_F03	CCATCCTTCGCTCGATTCCA	200		
	Bufo bufo	Bufbuf_cytb_R03	CGAGGGGTCGGAACATAAGG	1200		
	Bufo bufo	Bufbuf_cytb_P03	TTCTCTTCCTCATGCCCCTGCTCCATA	300	40417.7	126
Anura, Bufonidae	*Bufo calamita*	BufcalCBL_0390	GGGCTATGTCCTCCCGTGA	200		
	Bufo calamita	BufcalCBR_0490	TTGAACAAGCTCGGTCCCAA	1000		
	Bufo calamita	BufcalCB.prob0440	TTACAAACCTCCTCTCCGCC	450	61671.0	100
Anura, Bufonidae	*Bufo viridis*	BufvirCBL_0653	AAATCCCATTCCATGCTTACTACT	1200		
	Bufo viridis	BufvirCBR_0752	AAGAGGTTGGGGGCAAATGT	200		
	Bufo viridis	BufvirCB.prob0718	AGAAGGCTAGTATGAGTGCAAATCCA	450	61658.3	100
Anura, Hylidae	*Hyla arborea*	HylarbCBL	GACCTCCCTGCACCATCTAA	200		
	Hyla arborea	HylarbCBR	GAACAACCCAGTTGCGATTT	1000		
	Hyla arborea	HylarbCB.probe	TGGCTCCCTACTCGGAGTTTGCC	350	57338.3	93
Anura, Pelobatidae	*Pelobates fuscus*	PelfusCBL	TACTTGAATCGGAGGCCAAC	200		
	Pelobates fuscus	PelfusCBR	GAGGGGGATGAGGATAAGGA	800		
	Pelobates fuscus	PelfusCB.probe	TGGCCAACTGGCCTCCCTAGTC	450	61661.1	100
Anura, Ranidae	*Pelophylax ridibundus*	Pelrid_cytb_F01	TCCACACCTCCAAACTACGC	200		
	Pelophylax ridibundus	Pelrid_cytb_R01	AGAGTCCGGAAGCGATTTGG	600		
	Pelophylax ridibundus	Pelrid_cytb_P01	TACAGCCATCCTCACATGAATTGGCGG	250	94419.2	153
Anura, Ranidae	*Rana arvalis*	Ranarv_cytb_F04	GCCGCGACGTTAATAATGGC	200		
	Rana arvalis	Ranarv_cytb_R05	GTCTTGGCCGATGTATGGGG	1000		
	Rana arvalis	Ranarv_cytb_P04	CCACATCGGACGAGGCCTTTATTACGG	300	166027.0	269

(Continues)

TABLE 1 (Continued)

Order, family	Target species	Primer (F and R) and probe name (P)	Sequence, primer and probe 5′ → 3′ direction, with FAM and BHQ1 modifications	Optimal primer−/probe concentration per individual qPCR reaction (nM)	Molecular weight of dsDNA (Da)	Target fragment length (bp)
Anura, Ranidae	*Rana dalmatina*	RandalCBL_0546	CCACTTTATTCTCCCGTTTATC	200		
	Rana dalmatina	RandalCBR_0653	TTGTCTAGGCTGGAGTTAAG	1200		
	Rana dalmatina	RandalCB.prob0600	TCTCCACCAAACAGGATCGTC	500	66604.4	108
Anura, Ranidae	*Pelophylax lessonae*	Ranles_cytb_F01	TCCTCGGAGACCCAGACAAT	200		
	Pelophylax lessonae	Ranles_cytb_R01	GGGTCTTCTACAGGTTGGCC	600		
	Pelophylax lessonae	Ranles_cytb_P01	ATGTTCCGCCCTATCACCAAAGCACTC	300	180266.0	292
Anura, Ranidae	*Rana temporaria*	Rantem_cytb_F01	CTATCGCCCACATCTGTCGG	200		
	Rana temporaria	Rantem_cytb_R03	TAAAGGCCCCGTCCGATATG	1000		
	Rana temporaria	Rantem_cytb_P02	TCATGCCAATGGTGCATCATTTTTCTT	300	70930.1	115
Urodela, Salamandridae	*Ichthyosaura alpestris*	Ichalp_cytb_F04	CATCAACGGGGCCTCATTCT	200		
	Ichthyosaura alpestris	Ichalp_cytb_R04	ACGGGATGGCTGAGAGTAGA	1000		
	Ichthyosaura alpestris	Ichalp_cytb_P04	TTATCTTCACATTGGACGGGGCTTGCG	300	131979.7	215
Urodela, Salamandridae	*Lissotriton vulgaris*	Lisvul_cytb_F01	TTACCCGATTCTTCGCATTC	400		
	Lissotriton vulgaris	Lisvul_cytb_R01	CGGTTTGGTGAAGGAACAGT	1000		
	Lissotriton vulgaris	Lisvul_cytb_P01	ATTCCCCTTTCTTATTGCCGGG	500	53629.9	87
Urodela, Salamandridae	*Triturus cristatus*	TricriCB-L	GCAACACTCACCCGATTTTT	200		
	Triturus cristatus	TricriCBR	CCCCGTTTGGTGTAAGAAGA	1000		
	Triturus cristatus	TricriCB.probe	TGCATTCCACTTCCTGTTCCCATTC	350	59190.6	96

cristatus in the United Kingdom (Biggs et al., 2015). In our current study, we tested the same approach on 14 different amphibian species simultaneously.

2 | METHODS

2.1 | Initial de novo sanger sequencing of amphibian mitochondrial gene fragments

All the initial in silico design, PCR, and validation and optimization of primers and probes were carried out by expert users in a laboratory with uni-directional workflow and laminar flow hoods for PCR preparation to minimize the risk of cross-contamination between samples and reaction. The students were not involved at this stage. Our setup here makes use of two separate laboratories. One laboratory is used by professional researchers dedicated to eDNA work, and a second laboratory for high school students, also dedicated to eDNA work, located over 2 km away from the first laboratory. Trained professional researchers started in the first laboratory by obtaining DNA sequence data, and consulting the taxonomy of amphibians. The taxonomy of European species of amphibians is often revised and can lead to confusion. In the present study, we follow the taxonomy and nomenclature of Speybroeck et al. (2010) and Dubois and Bour (2010). Not all amphibian species found in Denmark are represented by sequences of mitochondrial DNA (mtDNA) for cytochrome *b* (*cytb*) and cytochrome oxidase 1 (*co1*) at the National Center for Biotechnology Information (NCBI) database. To allow for efficient primer and probe design, initial PCRs were performed on genomic DNA extracted from tissue samples and used for de novo sequencing of fragments of mtDNA-*cytb* and mtDNA-*co1*. The extractions of genomic DNA from tissue samples were performed with the Qiagen Blood and Tissue kit (cat. no. 69506) following the supplied protocol. The first PCR was set up with the aim of doing de novo sequencing using primers targeting a mtDNA fragment larger than 500 bp, either targeting *cytb* (L14841_CYB: 5′-AAAAAGCT TCCATCCAACATCTCAGCATGATGAAA-3′ and H15915_CYB: 5′-AACTGCAGTCATCTCCGGTTTACAAGAC-3′) (Kocher et al., 1989; Irwing et al., 1991) or *co1* (FishF1: 5′-TCAACCAACCACAAAGACAT TGGCAC-3′, FishF2: 5′-TCGACTAATCATAAAGATATCGGCAC-3′, FishR1: 5′-TAGACTTCTGGGTGGCCAAAGAATCA-3′, FishR2: 5′-ACTTCAGGGTGACCGAAGAATCAGAA-3′) (Ward et al., 2005). Each PCR tube was set up in triplicate reactions to have 25 μL reaction volumes comprising 1.25 μL forward primer (10 μM), 1.25 μL reverse primer (10 μM), 0.1 μL of AmpliTaq Gold Polymerase (5 U/μL), 2.5 μL dNTPs (2 mM per dNTP) (GeneON), 2.5 μL 10 × Buffer (AmpliTaq, GeneAmp), 1 μL $MgCl_2$ (25 mM), 14.4 μL ddH_2O and 2 μL of DNA template extracted from tissue (Table S1). Thermocycler (Applied Biosystems TM 2700, Denmark) conditions were set to have an initial 2 min preheat at 95°C, with 40 cycles comprising three temperature steps (95°C for 30 s, 45–56°C for 30 s, and 72°C for 90 s), followed by a final extension at 72°C for 5 min. Products were visualized in gel electrophoresis using a 2% agarose gel (SeaKem) and stained with GelRed (VWR Life Science). Successful amplicons were sent to Macrogen Europe (Amsterdam) for PCR cleanup and subsequent Sanger sequencing in both directions. Sequence reads were assembled, visually inspected, and manually corrected in Geneious vR7 (Kearse et al., 2012) to produce consensus sequences that could be used for designing specific oligos. Sequences obtained are available through NCBI GenBank (accession numbers: OQ130158-OQ130165).

2.2 | Design of primers and probes

The design and test of oligos follow previous protocols (Agersnap et al., 2017; Knudsen et al., 2019). Sequences of mtDNA *cytb* and *co1* genes from multiple representatives of the target and the non-target species were downloaded from the NCBI GenBank database and aligned with MAFFT v6.822 (Katoh & Toh, 2010) together with sequences obtained by de novo sequencing in Geneious vR7 (Kearse et al., 2012). Variable regions in the alignments were identified by visual inspection and suggestions for primers and probes were found with Primer3 v0.4.0 (Koressaar & Remm, 2007; Untergasser et al., 2012). The settings used in Primer3 for oligos were a primer length between 19 and 25 nucleotides, a melting temperature (Tm) between 58 and 63°C, with an optimal length of 20 nucleotides, and 60°C Tm. For the internal probe, a Tm in the range of 63–72°C, and a length between 23 and 32 nucleotides was set with an optimal length of 27 nucleotides and a Tm of 68°C. The limitation on the fragment length was to range from 70 to 400 nucleotides. Primer3 was used on only a single sequence from the target species, which does not consider the non-target species. Five of the suggested primer pairs and their internal probes were then imported into Geneious vR7 and mapped onto target sequences, that were aligned with MAFFT for further comparison with other representative sequences from target- and non-target species. Mapped primers were then visually inspected to check which oligos would anneal to unique regions for the targeted species and then compared with diversity available through NCBI GenBank with Obitools (Boyer et al., 2016). Oligos evaluated as being unspecific were not given any further consideration. Four to five of the specific primers, suggested by Primer3, were then ordered for each species to test in initial polymerase chain reaction (PCR) setups. We did not order probes at this stage, as internal probes cost more than primers, and if the primers fail to amplify the target region, it is irrelevant whether the internal probe has a high affinity toward the target region. A matching internal probe oligo was only ordered for the primer combinations which returned a positive amplification for the targeted species. The specificity of the primers might in itself have been adequate for doing the monitoring with simple PCR or in qPCR using SYBR Green, but we decided to also use internal probes to avoid having students doing gel electrophoresis, and to have an extra level of specificity with an internal hybridizing probe.

2.3 | Assay design

Genomic DNA was extracted from tissue samples from the target species and from co-occurring amphibian species (Table 1) by using the Qiagen Blood and Tissue kit (cat. no. 69506). In the first laboratory, a range of PCR setups were then prepared by trained professional researchers, using the five specific primer sets inferred with Primer3. These PCR setups were done individually in parallel for each of the species targeted in this study, with multiple combinations of the different specific primers for each species targeted, as long as the resulting target fragment was shorter than 400 bp (Text S1). Many of these in silico-suggested primer pairs turned out to be unspecific when compared in sequence alignments or failed to amplify the target species. Because of this, we have not listed all these initial Primer3 suggestions, but only listed the primer pairs that we also could validate and use for specific monitoring.

Using the primers that were successful in the initial PCR, we prepared the first qPCR setup with the inclusion of a probe with a 5' FAM-dye and a 3′ black hole quencher-1 (BHQ-1) modification (Smith & Osborn, 2009) (Table 1). This first qPCR was set up to run on a Stratagene Mx3005P and prepared with 25 μL total reaction volumes comprising 10 μL of TaqMan Environmental Master Mix 2.0 (Life Technologies), 10 μL ddH_2O, 1 μL of each primer (forward and reverse) (10 μM each), 1 μL of probe (2.5 μM) and 2 μL of DNA from tissue (Table S1). Using only 10 μL of TaqMan Environmental Master Mix 2.0 was preferred to ensure the cost of setups could be minimized. The temperature settings were set to have an initial preheat at 50°C for 5 min, and 10 min at 95°C, followed by 50 cycles at 95°C for 30 s and 60°C for 1 min, with fluorescence collected at the endpoint in the final 1 min 60°C step. The initial preheat step at 50°C for 5 min was included in case later setups required the addition of Uracil-DNA Glycosylase (UNG) to degrade any cross-contamination from previous qPCRs. We never experienced cross-contamination, but kept this step throughout all qPCR setups, to avoid deviating from the original setup. No template controls (NTC) were prepared in two replicates to check for any contamination. Data files were exported from the MxPro software and analyzed with R v4.3 (R Core Team, 2023). The optimal primer and probe combination was selected from the earliest onset of amplification and highest relative fluorescence levels. Extractions of genomic DNA (Table S1) were not standardized to an even concentration level for all extractions. This can be considered as an inadequate test of the specificity of the assays, but we used 50 amplification cycles in the qPCR setup to allow for even low concentrations of non-target species to amplify if oligos had affinity for the target region. Also, the genomic tissue extractions will be at much higher concentration levels than what a filtered water sample can ever return from in vivo tests.

2.4 | Assay optimization

A second qPCR was prepared to infer the optimal concentrations of the inferred specific primers in the qPCR tubes. This second qPCR included two replicates of each combination of concentrations, with 25 μL total volume reactions comprising 10 μL of TaqMan Environmental Master Mix 2.0 (Life Technologies), 2 μL ddH_2O, 1 μL of probe (2.5 μM) and 2 μL of DNA extracted from tissue added, and 5 μL of each primer (forward and reverse, in variable concentrations at 1, 2, 3, 4, 5, or 6 μM) to get final concentrations in each reaction of each primer to be 0.2, 0.4, 0.6, 0.8, 1.0, or 1.2 μM. The volume of added primer is in this way changed from previous setups, but this ensures that variable final reaction concentration per reaction tube can be tested. The Stratagene Mx3005P machine was set to have the same temperature settings as applied in the first qPCR. The resulting data were analyzed in R v4.3 (R Core Team, 2023) (Figures S5–S11). The optimal concentration of primers (Table 1) was selected based on the earliest amplification at onset. Once the optimal concentration of primers was inferred, an optimal concentration of the hydrolysis probe was determined in a third qPCR. This third setup with two replicates also used 25 μL total volume reactions comprising 10 μL of TaqMan Environmental Master Mix 2.0 (Life Technologies), 2 μL ddH_2O, 1 μL of each primer (forward and reverse) in the optimal concentrations inferred in previous qPCR, 3 μL of template DNA, plus 8 μL of the matching probe in variable concentrations at 0.31, 0.63, 0.94, 1.25, 1.56, 1.88, 2.19, or 2.50 μM to get final concentrations in each reaction of the probe of 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, or 0.8 μM. The time and temperature settings used were the same as in the two previous qPCR setups described. Again, the optimal concentration was selected from the earliest onset of amplification determined by the lowest cycle of quantification (Cq) threshold and the highest relative fluorescence.

2.5 | Establishing limit of detection (LOD)

Using a polymerase with exonuclease activity in a third PCR setup with the primers found for each species (Table 1) made it possible to prepare PCR products that could be purified, diluted, and used as standard positive absolute controls in the following qPCR. This third PCR setup was prepared in 50 μL reaction volumes with 38.5 μL ddH_2O, 5.0 μL × 10 buffer (standard including $MgCl_2$), 2.0 μL dNTP (2 mM of each dNTP), 0.5 μL (5 U/μL) AccuPol DNA proofreading polymerase (AccuPOL DNA polymerase, Ampliqon, VWR # 733–1324), 1.0 μL of genomic DNA from the target species, and 1.0 μL of each primer (forward and reverse) (10 μM). Temperature settings were 95°C for 2 min, followed by 30 cycles of 95°C for 30 s, 55°C for 30 s, and 72°C for 1 min, and a final extension at 72°C for 5 min, with a hold step at 10°C until tubes were removed from the thermocycler. From each reaction, 5 μL of the resulting PCR were used to check products in a 2% agarose gel. Successfully amplified products were cleaned with the Qiagene PCR clean-up kit, by adding 55 μL ddH_2O to the remaining 45 μL in the PCR reaction, making the total volume 100 μL, and then following the supplied dsPCR clean-up protocol. Concentrations of the purified dsPCR-amplicons were measured on a Qubit 2.0 Fluorometer (ThermoFisher Scientific) using the QubitTM dsDNA High Sensitivity kit. The molecular weight of the

target fragment was found with the OligoCalc calculator webpage (Kibbe, 2007) and used to calculate the number of copies per µL, as in previous studies where absolute standard dilution series are used as positive controls (Agersnap et al., 2017; Knudsen et al., 2022). For each assay, the dsPCR target fragment was stored at −20°C in a concentration of 1E+6 copies per uL and only thawed again when used for preparing a dilution series. The dilution series was prepared on the very same day prior to running the qPCR test for inferring limit of detection (LOD) and limit of quantification (LOQ).

2.6 | Determining the lowest level of detection from a dilution series

A fourth qPCR was performed with the settings as before, using the optimal concentrations inferred for oligos and adding the purified positive control as an absolute standard dilution series. The dilution steps were prepared in three replicates and in tenfold decrementing steps spanning from 1E+5 copies/µL to 1E+0 copies/µL. For each assay, three negative target controls (NTC) were added, and two additional positive controls representing 10-fold dilutions of the original genomic DNA extracted from tissue. Results of the standard curves were exported from the MxPro software and analyzed using R v4.3 (R Core Team, 2023) in Rstudio v2023.03.1 (RStudio Team, 2023). With three technical replicates per dilution level step, the LOQ was determined as the lowest concentration at which all three standard dilution series replicates were able to amplify on the target fragment. The LOD was determined as the lowest concentration where at least one replicate in the standard dilution series was able to amplify (Table 2, Figures S19–S25). These rough definitions of limitations are in line with how limitations have been inferred for previously published species-specific assays (Agersnap et al., 2017; Knudsen et al., 2019). The LOD levels (Table 2) were also inferred with the R code developed in previous studies (Klymus et al., 2019; Merkes et al., 2019). All of the above PCR setups and validation tests were performed by a professional trained researcher in a laboratory with a uni-directional workflow, that separates pre- and post-PCR related work, and the PCR setups were prepared in laminar flow hoods. This laboratory is dedicated to work with eDNA and ancient DNA, and this laboratory is sterilized every night with UV light and is located more than 2 km away from the laboratory where the students perform the test on their water samples.

2.7 | Citizen science collection of water samples and extraction from filters

From 2017 to 2019 a total of 115 freshwater samples were collected by high school students in Denmark who volunteered to be a part of the DL project (Tøttrup et al., 2021). An additional nine samples were collected from 2019 to 2022 under separate projects, but also analyzed by students attending the DL laboratory. Students collected eDNA samples from a variety of freshwater environments widely distributed within the national borders. To optimize the chance of

TABLE 2 Limit of detection (LOD) and limit of quantification (LOQ) for the species-specific assays targeting amphibian species. The LOD and LOQ limits reported here are only based on the three technical replicates per dilution level, and additional tests with more replicates are required in order to evaluate more precise levels of LOQ and LOD for these assays. All values were inferred from the standard curves in the supplementary material. The highest Cq at LOD is obtained from the latest amplifying replicate at LOD. The intersecting (Inters) Cq at LOD is where the standard curve crosses the LOD. The intersection between the standard curve and 1E0 is "Inters1." Amplification factor "Ampl Factor," and the "slope" of the standard curve. The "Efficiency" is calculated as "(−1 + (10^(−1/slope))) × 100".

Species	Common name	Highest Cq at LOD	Inters Cq at LOD line	LOD (copies/µL)	LOQ (copies/µL)	Efficiency (%)	Ampl factor	Inters1	Slope
Bufo bufo	Common toad	40.61	40.016	30	30	75.26	1.753	46.078	−4.10
Bufo calamita	Natterjack toad	40.03	38.977	30	30	89.33	1.893	44.306	−3.61
Bufo viridis	European green toad	39.62	39.901	30	30	96.80	1.968	44.925	−3.40
Hyla arborea	European tree frog	39.63	39.117	30	30	78.36	1.784	44.995	−3.98
Ichthyosaurus alpestris	Alpine newt	42.07	41.366	3	30	105.14	2.051	42.895	−3.20
Lissotriton vulgaris	Northern smooth newt	38.95	42.733	3	30	80.24	1.802	44.598	−3.91
Pelobates fuscus	Common spadefoot	41.14	40.629	3	30	102.86	2.029	42.182	−3.26
Pelophylax ridibundus	Marsh frog	42.51	40.375	3	30	77.35	1.773	42.292	−4.02
Rana arvalis	Moor frog	44.23	37.335	3	30	105.39	2.054	38.861	−3.20
Rana dalmatina	Agile frog	39.94	39.509	30	30	87.84	1.878	44.904	−3.65
Rana lessonae	Pool frog	33.22	37.678	3	30	135.77	2.358	38.959	−2.68
Rana temporaria	Common frog	40.98	40.632	3	30	92.28	1.923	42.312	−3.52
Triturus cristatus	Great crested newt	41.14	39.075	30	30	99.78	1.998	43.989	−3.33

detecting eDNA from the 14 target amphibian species in Denmark, students were instructed to carry out sampling between early May and June in the mating season and also from June to late September, when most amphibians interact with their aquatic habitat. However, school summer holidays limited the sampling frequency from late June onwards. Each participating group of students received the necessary sampling instructions and equipment in advance; including a 50 mL syringe, two Sterivex filter cartridges (Milipore), plugs for both ends of the filters, a tube with 2 mL 96% ethanol, a 5 mL syringe for adding ethanol to the filter and instructions on how to carry out the sampling, filtering of water and how to add ethanol to the filter. Although an immediate frozen Sterivex filter has been shown to be slightly better at preserving eDNA than adding a buffer or ethanol (Spens et al., 2017), we opted for a solution where students added 2 mL 96% ethanol per filter and closed each filter with small screw threaded plugs, since the samples were to be returned by postal service. This simplified the storage and transportation of the filters and meant that the schools could return their samples to the museum by common mail service. Students were instructed to note the additional information on the sampling site, including the date, position in latitude and longitude, volume of water filtered, surface area of the water body sampled, and other observations made during sampling. Filters received at the NHMD were extracted by laboratorial trained staff using the Qiagen Blood and Tissue kit. The filters were emptied of ethanol and dried out. The ethanol was then centrifuged at 8000 rpm for 20 min, and the supernatant ethanol was discarded, the pellet was then resuspended in 720 µL ATL buffer. The 720 µL ATL with resuspended pellet was then transferred to the dry filter, 80 µL of proteinase K was added to the filter, and the ends were closed with luer-lock plugs. Filters were then incubated at 56°C overnight, extracted the following day, and eluted in 200 µL volume of AE buffer. Extraction of eDNA from filters follows previous protocols (Sigsgaard et al., 2016; Spens et al., 2017) apart from the last step where 200 µL AE buffer was added for the final elution step. All resulting extractions were then stored at −20°C until the high school students visited the NHMD and performed a qPCR analysis on around 170 µL of the extraction. The remaining 30 µL of extracted DNA was stored for later retesting.

2.8 | Detection of eDNA performed by students and mapping eDNA from amphibians

Up until this stage, all prior laboratorial work had been carried out by trained researchers in the first eDNA-dedicated laboratory. The following final qPCR detection of amphibian eDNA on the water samples was performed by the students in the second laboratory. This screening of the samples made use of the optimized qPCR protocol (Text S1). We decided for educational purposes to alter the plate setup for qPCR to be different from a more conventional setup that focuses on just a single species and includes a standard dilution series, to instead be a specialized setup that makes it possible for each class of students to test multiple assays on their own water sample (Figure 1). The assays had not yet been tested in multiplexing setups, and we decided for this plate setup as we wanted each class of students to use all 14 assays simultaneously in a single qPCR setup, instead of only using a single assay per qPCR setup (Figure 1, Text S2).

All data files from each qPCR run were named with a standardized filename that specified the collection date for the water sample, the unique number of the individual water sample and the analysis date, and then using a Unix code all results were merged to one file with all data prepared by the students. Results were only considered valid if a set of four wells had negative and positive controls returning the expected results (Figure 1). If either or both of the controls failed to perform as expected, the test was considered to be a "failed test" and all results from this test were disregarded. *Pelophylax lessonae* is not known to occur as a native species in Denmark (Fog et al., 2019) and any detections for *P. lessonae* were interpreted as being indications of the presence of *Pelophylax* sp. Species detections were then mapped independently using R v.4.3 (R Core Team, 2023) and compared with observations obtained from iNaturalist (Ueda, 2021) limiting records to "research grade," the Global Biodiversity Information Facility (GBIF.org, 2022), "https://arter.dk" (Møller et al., 2020) and expert opinions (MH and PFT). Any inconsistencies between the students' approved positive eDNA monitoring and conventional recordings, were subsequently checked with an additional qPCR on the 30 µL residual subsample of the identical extraction from the filtered sample originally collected and analyzed by students and hereafter kept at −20°C. This was performed by the first author, but with the inclusion of a standard dilution series of the target amplicon in four replicates, in seven 10-fold dilution steps, and the extracted water sample was tested in eight replicates.

Successful monitoring test sets of four wells performed by students were also compared with the surface area of the water body sampled. The area of the water body was provided by each high school class attending the course, who inferred this surface area using internet maps, which may be biased by students' miscalculations. To check whether the area of water body sampled was normally distributed we performed a Shapiro–Wilk test (Shapiro & Wilk, 1965) for normality in R v4.3.

3 | RESULTS

3.1 | Primer and probe specificity and optimization

The in vitro test on genomic DNA from congeners confirmed specificity in the primers to a "level 2" (Langlois et al., 2020; Thalinger et al., 2021). The tests on concentrations of the oligos allowed for an increased sensitivity in the detection of eDNA (Text S3). The different species-specific qPCR assays showed different LODs (Table 2). The slope of the standard curve for each assay could also be determined to infer the efficiency of each assay. For four of the assays developed the efficiency climbs above 100%, which could be an artifact caused by the presence of primer dimers or polymerase inhibition. Six of the assays had an efficiency below 90%, which can

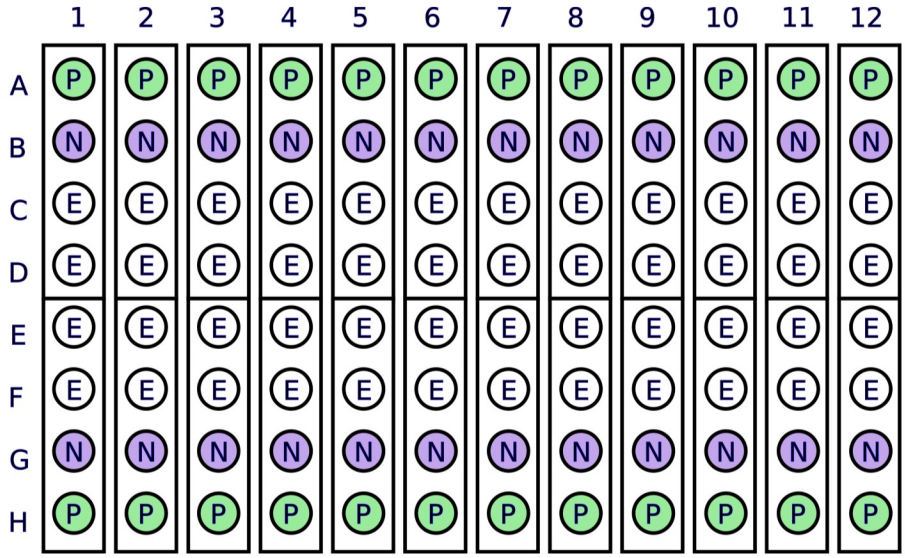


FIGURE 1 Arrangement of setup for qPCR test performed by high school students. Each set of four tubes was prepared as a master mix to detect one specific species. Multiple species of amphibians could in this way be detected simultaneously. The very same water sample that the class had collected prior to their visit was subdivided into 25 aliquots, allowing for detecting amphibians in 24 individual mixes, and one residual aliquot that could be retested later on by a professional researcher. A set of four tubes comprised a (P)ositive control, a (N)egative control, and two tubes with (E)xtraction from the filtered water sample.

be caused by low affinity between the oligos and the target fragment. The standard dilution series and the resulting standard curves indicated that all assays can detect as low levels of eDNA as three or 30 molecules in each qPCR reaction well (Table 2).

3.2 | Obtained samples and success rate for the citizen science detections

The extracted water samples allowed for the screening of the 14 species of amphibians in 2551 sets of four qPCR tubes (Figure 1) across Denmark (Figure 2). Each set comprised one tube for a positive control, one tube for a negative control, and two qPCR reaction tubes containing extracted water sample. In 921 (36%) of the 2551 sets prepared by the students, the positive and negative controls could not be approved, and were regarded as "failed tests" (Table 3, Figure S30). These 921 failed results were discarded straight away and not used for further data analysis. In the 1639 sets that could be approved, that is, the 1639 sets with both the positive and negative control behaving as expected, 1443 sets returned no detection of eDNA in the extracted water sample, and 196 sets returned detection in one or two out of two qPCR tubes with extracted water sample in the set. Among the 196 sets with positive and approved detections, there were six test sets that returned detection of *B. calamita*, *P. fuscus*, and *R. dalmatina*, but outside the area of known occurrence for these species. Using the residual extractions for these six tests, a trained professional researcher performed a more detailed test for each of these three species to check if the first results obtained by students could be replicated, but this time the researcher included more technical replicates of both the extractions and the standard dilution series. The inclusion of both positive and negative controls in the qPCR setups performed by the students reduced the risk of drawing incorrect conclusions from false positive detections. Six of the common species of amphibians, that is, *B. bufo*, *L. vulgaris*, *Pelophylax* sp., *R. arvalis*, *R. temporaria*, and *T. cristatus* monitored by eDNA could in this way be mapped across the sampling area (Figures 2 and 3, Figures S31–S34) after removing failed qPCR attempts (Figure 1, Figure S31). Another three species, that is, *B. calamita*, *P. fuscus*, and *R. dalmatina* were detected but only rarely (Figure 2, Table S2). Findings were compared with records obtained from "https://arter.dk" (Figure 3), iNaturalist (Figure S33), and GBIF (Figure S34), with eDNA detections for some of the species confirming findings from these data portals. For rare species such as *B. bombina*, *B. calamita*, *B. viridis*, *H. arborea*, *I. alpestris*, and *R. dalmatina* many tests returned no detection of eDNA for the water samples collected within the area of known occurrence (Figure 3, Figures S33 and S34).

Comparison of students obtained eDNA results with relatively well-known amphibian distributions only found two odd out-of-range occurrences of *B. calamita* in Eastern Denmark, two detections of *P. fuscus*, and two detections *R. dalmatina* in northeastern Denmark. Reanalysis of these six samples by the first author, with the inclusion of standard dilution series and eight replicates of the extracted water sample, failed to reproduce these unlikely detections. Because of this, we removed these detections from further analysis. This suggests that a proportion of the approved positive detections within the area of known occurrence could be false positives. However, as only six sets out of 2551 sets were suspected of being false positives outside the range of the known distribution, we assume that an equally small proporption of the approved positive detections inside the area of known occurrence also could be false positives.

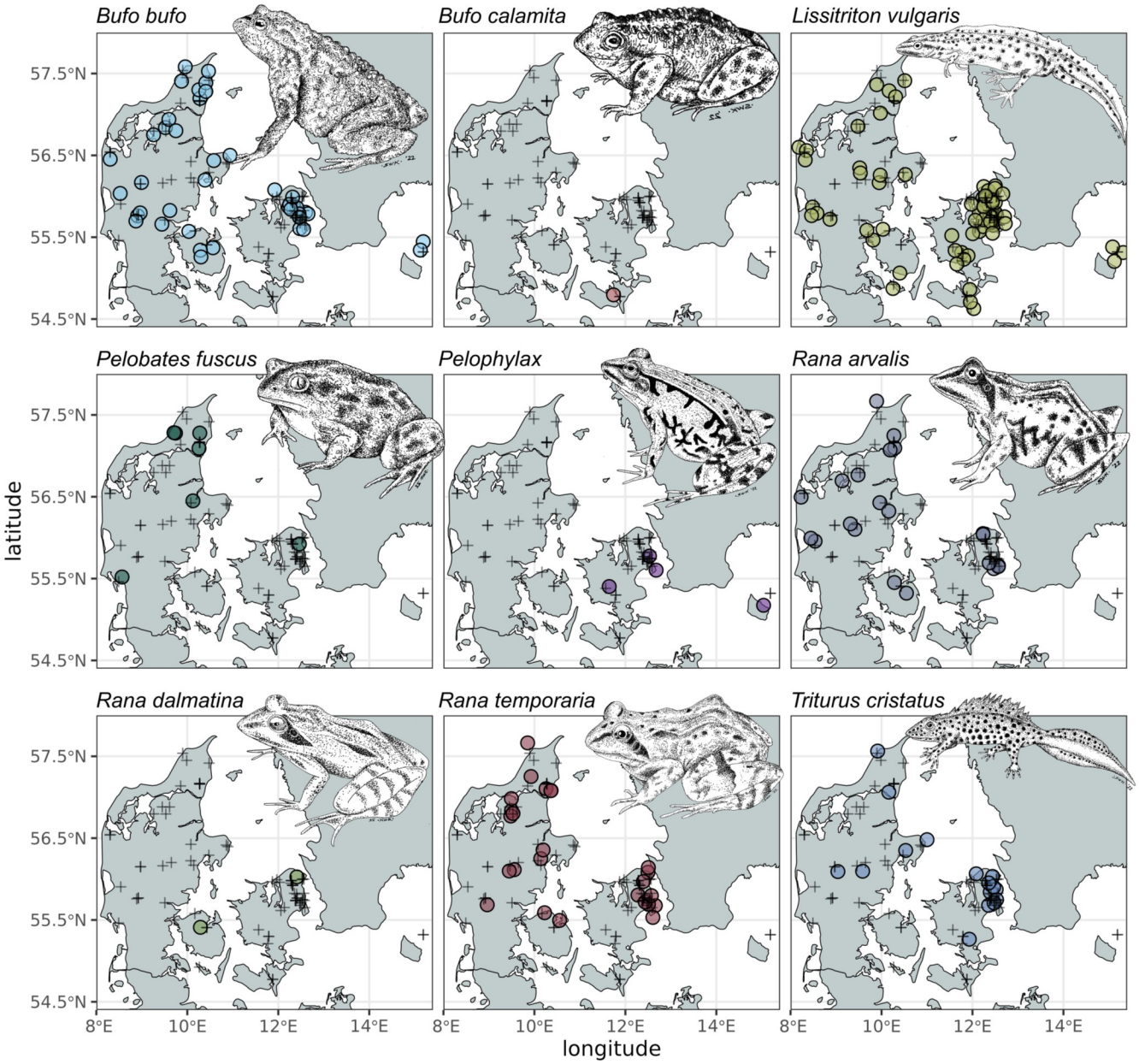


FIGURE 2 Geographic representation of eDNA detected for nine species of amphibians. No eDNA was detected for *Bombina bombina*, *Bufo viridis*, and *Ichthyosaurus alpestris*, which is why these species are not included here. Only successful detections in at least one out of two replicates in a test set of four tubes are included. Crosses indicate sampling locations. Colored points indicate positive eDNA detections. All qPCR tests are plotted on maps in Figures S31–S35.

3.3 | Monitoring eDNA from amphibians in filtered water samples

The efficiency (Table 2) of a given primer/probe system (Table 1) together with the proportion of non-approved attempts (i.e., failed attempts in Table 3), can be interpreted as an indication of how often the untrained students will end up with non-approved analyses and thereby be incapable of producing a successful outcome. With 54%, the rate of false positives for the qPCR assay targeting *Pelophylax* sp. was higher than for any other of the assays (Table 3, Figure S30). The application of the assays for the detection of eDNA from *B. calamita*, *R. dalmatina*, and *P. fuscus* resulted in scarce and limited detections, but still within their known distribution (Figure 3, and Figures S33 and S34).

Comparing the eDNA detections with records from "https://arter.dk" (Figure 3) and GBIF and iNaturalist (Figures S32–S34) resulted in a few odd records from these databases, which most likely caused by users having deposited incorrectly identified records.

Sampling time was within the aquatic period of the amphibians, with early detections in late April for the common species (*B. bufo*, *L. vulgaris*, and *T. cristatus*), but failed to cover the late summer season due to school summer holidays (Figure S36). Comparison of the area

TABLE 3 Accumulation of individual analysis sets of four qPCR tubes performed by students. A qPCR analysis of a given water sample is approved (Approv) when the included negative control tubes and the positive control tube both returned the expected result, if not, the analysis is non-approved and considered a failed test. If the analysis shows a positive eDNA signal in at least one of the two other tubes per set of four tubes, the eDNA from the corresponding species is present and detected in the water sample. "Failed tests" are sets that are considered non-approved analyses, "Approv sets, no eDNA" are sets of approved analyses where no eDNA was detected, "Approved analyses sets, eDNA detected" are sets of approved analyses where eDNA was detected, "Total number of attempted sets" is the total number of sets of four qPCR tubes attempted analyzed over 2017–2022.

Species	Common name	Sets that are failed tests, non-approved analyses	Sets that are failed tests, percentage	Approved analyses sets, no eDNA detected	Approved analyses sets, no eDNA detected, percentage	Approved analyses sets, eDNA detected	Approved analyses sets, eDNA detected, percentage	Total number of attempted sets
Bombina bombina	European fire-bellied toad	76	45%	94	55%	0	0%	170
Bufo bufo	Common toad	73	45%	46	29%	42	26%	161
Bufo calamita	Natterjack toad	42	24%	133	76%	1	1%	176
Bufo viridis	European green toad	43	24%	133	76%	0	0%	176
Hyla arborea	European tree frog	43	23%	142	77%	0	0%	185
Ichthyosaurus alpestris	Alpine newt	72	47%	81	53%	0	0%	153
Lissotriton vulgaris	Northern smooth newt	70	30%	89	38%	74	32%	233
Pelobates fuscus	Common spadefoot	54	29%	125	67%	8	4%	187
Pelophylax sp (a)	Marsh frog/ pool frog/ edible frog	165	54%	134	44%	6	2%	305
Rana arvalis	Moor frog	98	46%	92	43%	22	10%	212
Rana dalmatina	Agile frog	44	21%	165	78%	2	1%	211
Rana temporaria	Common frog	70	48%	52	35%	25	17%	147
Triturus cristatus	Great crested newt	62	26%	157	67%	16	7%	235
Total count		912	36%	1443	57%	196	8%	2551

Note: (a) Any eDNA detection of pool frog (*Rana lessonae*) is in this study considered to be confirmation of the presence of edible frog (*Pelophylax* kl. *esculentus*), as the assay target the mitochondrial genome in the parent species: pool frog, but only edible frog is known to occur in Denmark.

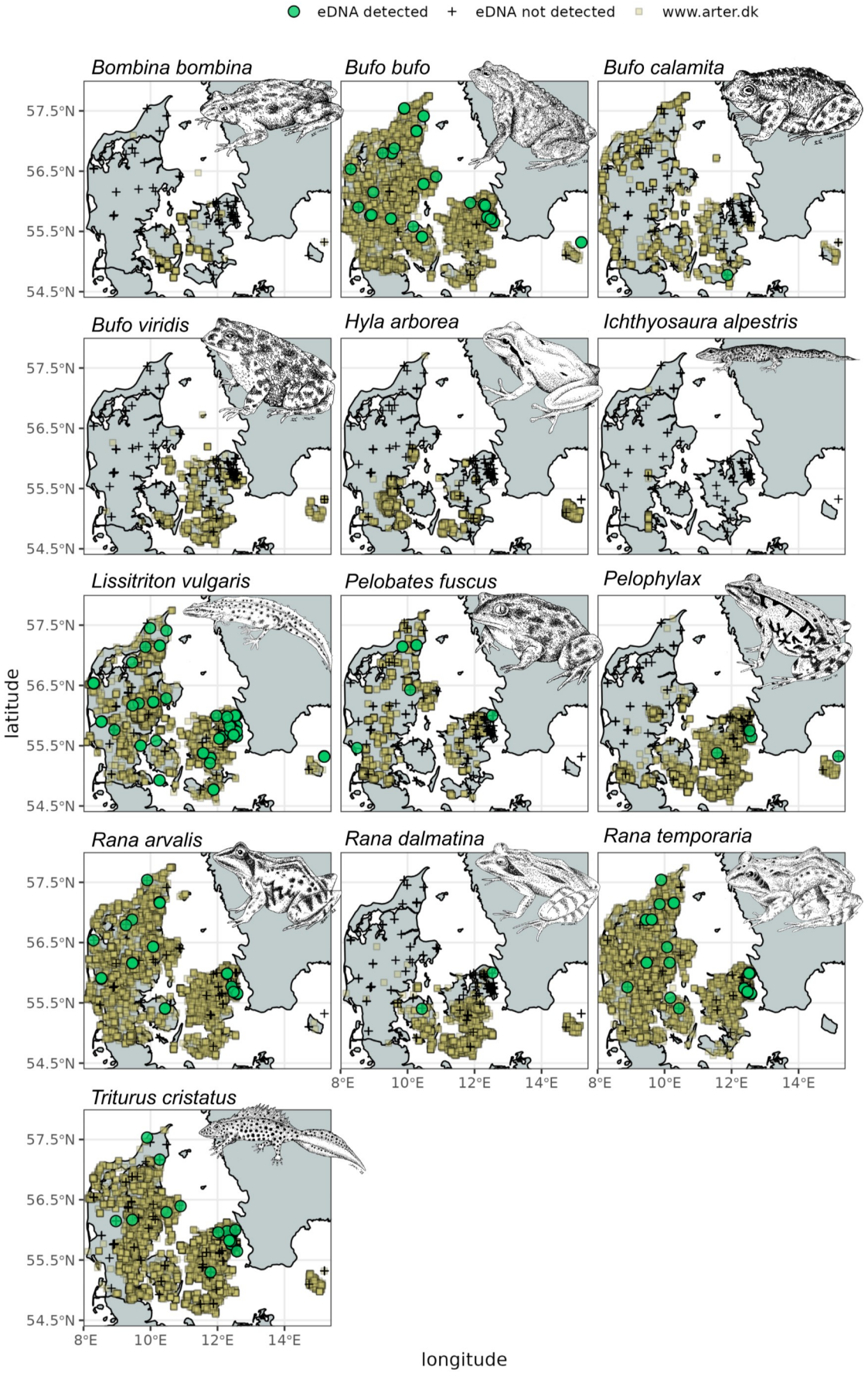
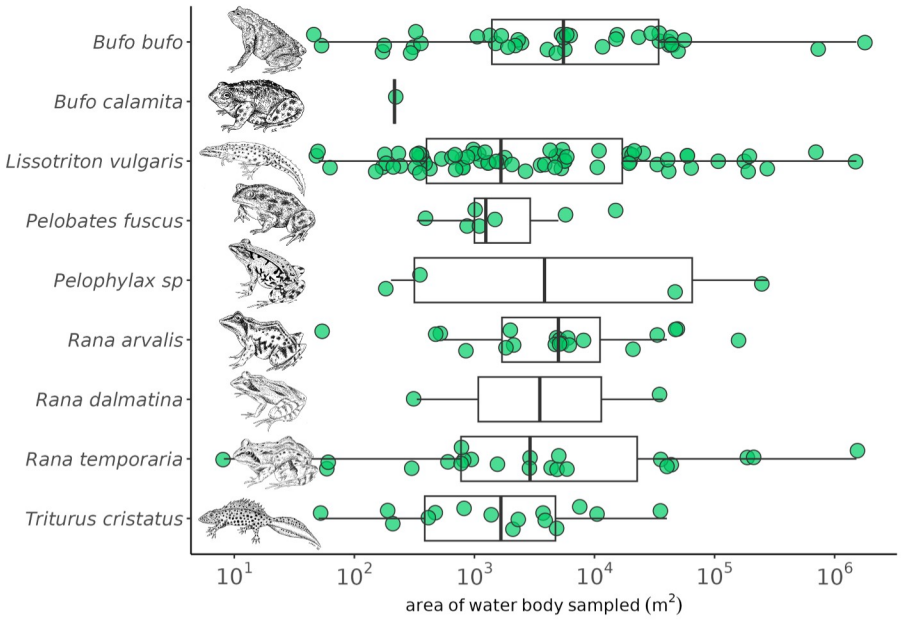


FIGURE 3 Geographic representations of records of amphibians in Denmark, reflecting both traditional monitoring and eDNA species-specific monitoring performed by high school students. The traditional monitoring reflects research grade records obtained from the "www.arter.dk" database (khaki colored squares). Approved positive detections of eDNA performed by students (green colored points) as well as approved zero detections (crosses) among the approved students' eDNA monitoring attempts. The "failed tests" have been excluded. Similar comparison with records obtained from GBIF and iNaturalist can be found in Figures S33 and S34. As in Figure S30 *Pelophylax ridibundus*, *Pelophylax esculentus*, and *Rana lessonae* have been grouped together under *Pelophylax* sp. Drawings are prepared by the first author.

FIGURE 4 Comparison of eDNA monitoring result and the surface area of the water body sampled. The result of the eDNA monitoring only considers the positive approved qPCR tests, excluding the "failed test" attempts, and excluding the approved negative detections. The water body area sampled is not normally distributed, and does not reflect that amphibians have a preference for a certain area of water body. As in Figure 2 *Pelophylax ridibundus*, *Pelophylax esculentus*, and *Rana lessonae* have been grouped together under *Pelophylax* sp. Drawings are prepared by the first author.



of the water body sampled and the eDNA detected showed an even spread of detections across the water body area (Figure 4). But both the sampling period and the area of the water body sampled are not normally distributed from the Shapiro–Wilk test performed (for both the sampling period and water body area the probability was above 0.05), which means these diagrams cannot be interpreted as representing the occurrence of these amphibians in time and size of the water body.

4 | DISCUSSION

Among the 14 qPCR assays we designed and validated 12 were found to be specific for the targeted species of amphibian, when compared with the genetic variation of northern European species of amphibians. The two assays targeting *P. ridibundus* and *P. lessonae*, were specific towards each of these two species (Figure S3), but are also capable of detecting the klepton species *P.* kl. *esculentus*. Since *P.* kl. *esculentus* carries the mtDNA from either *P. ridibundus* or *P. lessonae*, we could not make a mtDNA-based species-specific assay toward *P.* kl. *esculentus*, but using the assays towards *P. ridibundus* and *P. lessonae* in combination can at least help identify areas where these two parental species can hybridize. A high proportion of failed analyses for detecting *P. lessonae* could indicate that this assay perhaps has a relatively lower sensitivity towards the targeted species, which can result in more frequent failed qPCR analysis, which could mean that it might be hard to detect *Pelophylax* sp. using this assay. The assays presented here were all tested and validated to level 4 (Thalinger et al., 2021). However, our approach only used duplicate reactions per species per water sample. If these 14 assays are to be brought towards the operational level (level 5) in the investigated region (northern Europe and Scandinavia) more replicates need to be included per assay per locality sampled and monitored. Positive detection after professional application of the assays in the region we studied can be interpreted as the "target is very likely to be present" (Thalinger et al., 2021), but with more technical replicate analysis being a requirement.

4.1 | Evaluating the detections from previously known distributions

Various elements can influence the results obtained from the qPCR analyses. Rushed training of the students prior to preparation of the mix of reagents, and low motivation among students can result in an increased proportion of failed tests. The first two hours of the course were devoted to train students in using a pipette to avoid pipetting errors. Having the students analyze the very same water sample they collected themselves, were two measures taken to avoid too many incorrect mixes of reagents and to increase the motivation. Each water sample was analyzed at least once and occasionally twice for the amphibians targeted. Additional qPCR machines could have

allowed for parallel verification of the eDNA content in the water samples. Better equipment such as access to laminar flow hoods for the students could also have led to fewer failed tests.

The affinity of the primer/probe system towards the targeted genetic region will vary between assays. For both students and a trained expert, a low affinity toward a targeted region can result in a higher frequency of false negative detections. The rarity of some of the monitored species, for example, *B. bombina*, *B. viridis*, and *I. alpestris*, will influence the ability to produce authentic maps of the distribution of these rare species (Figure 3, Figures S32–S34). *Bufo bufo*, *L. vulgaris*, and *R. temporaria* are among the most commonly detected species across the water samples (Table 3, Figure S30), whereas eDNA from *B. bombina*, *B. viridis*, and *I. alpestris* were not detected in any water samples (Figure 3). These three species have the northern edge of their distributions in the eastern, southern, and central regions of Denmark and southern Sweden, and all three species are also rare in Denmark and only found at few sites in specific regions of the country (Fog et al., 2019) (Text S4). The best approach for increasing the chance of detecting the rare species is to increase the number of replicates and the frequency of sampling, but this goes for all kinds of monitoring of rare organisms.

4.2 | Applicability of the assays

The assays cannot be applied in central Europe without being validated with extracted DNA from congeners in the country they are to be used in prior to operational use. During assay design (in silico) it was shown that there is an insufficient number of mismatches in the 3′-end of the primers when compared with central European species of amphibians (Figures S26–S29). The in silico comparison of sequences obtained from NCBI GenBank included multiple representatives of the target species sampled from various regions of Europe. This indicates that the assays should pick up eDNA from the target species all over Europe. However, in some parts of Europe, the assays may also detect eDNA from sympatric congeners which were not tested in this study (Table S1). The genera: *Bombina*, *Bufo*, *Hyla*, *Pelobates*, *Pelophylax*, *Rana*, *Ichthyosaura*, *Lissotriton*, and *Triturus*, all include other European species which are evolutionary closely related to the 14 species targeted in this study. Hence, the assays presented here may detect eDNA from other sympatric congeners found in central and southern Europe, which were not included in our tests. Before making use of these assays outside Denmark it is advisable to perform some preliminary tests on DNA extracted from tissue from other non-target species, or at least make a comparison with the targeted gene sequence in other co-occurring species that potentially could give rise to false positive detection.

Bufo calamita is a rare species in Denmark and is today mainly associated with coastal sandy areas—as can be found mainly along the west coast of Denmark (Fog et al., 2019) (Figure 3, Figures S32–S34). The samples collected by students had limited coverage of this area, which is probably why this species was only detected once by eDNA. Students did initially detect *B. calamita* by eDNA in two water samples near Copenhagen in the eastern part of Denmark. However, professional reanalysis of these two water samples in eight replicates could not reproduce this observation. Consequently, these observations were removed from the dataset. Students' setup of qPCR also resulted in two eDNA detections of *Rana dalmatina* in the northwestern part of Denmark. Professional reanalysis of these two water samples could not reproduce these detections outside the known area of distribution for *R. dalmatina*. Consequently, these observations were also removed from the dataset. Involvement of students in the laboratorial tests and qPCR setup will, without doubt, lead to a few odd detections that are outside the known area of distribution for some species. Independent analysis performed in parallel by a trained researcher or laboratorial staff could help confirm or reject such odd detections. Over the two years of the DL project focusing on amphibians, only two species detections (*B. calamita* and *R. dalmatina*) in four water samples required this type of professional reconfirmation. More than 2550 test sets (with each test set comprising four tubes) were used to analyze 124 filtered water samples (Table 3) that was carried out by students over the two years. That six detections out of 2551 detection attempts had to be re-evaluated by a researcher shows that there is a risk of obtaining false positive detections. We were only able to identify these six detections as false positive because they fall outside the area of known occurrence. An equal proportion of false positive detections might have escaped our attention because they come from the area of already known distribution for these amphibians. Not being able to identify false positives inside the area of known distribution, is a limitation in our setup. Because of this, our citizen science approach cannot replace conventional monitoring, as our data makes it difficult to evaluate if the distribution of an amphibian species is diminishing. Still, a citizen science project as this may generate a large amount of valuable data and can be a valuable supplement to conventional monitoring. But careful evaluation of the results obtained is still required.

It is important to note that the distribution inferred here (Figure 3) is only valid for the specific freshwater environments where the water sampling took place. The qPCR results do not show a general distribution of all species across the country and could easily hold false negative results (e.g., due to sub-optimal sampling in August) or negative results from freshwater habitats not suitable for the targeted species.

4.3 | Benefits and challenges of citizen science in eDNA research

Making use of a citizen science approach to collect samples at a national scale provided a broad coverage of sampling sites suitable for testing the assays developed. Other eDNA studies also engaged the public with sampling (Agersnap et al., 2022; Julian et al., 2019)—for example, when monitoring nonindigenous (Secondi et al., 2016) or threatened species of amphibians (Villacorta-Rath et al., 2021).

Sampling of eDNA is straightforward and can be facilitated by instructional videos together with detailed and illustrated instructions (Agersnap et al., 2022) and supported by sampling gear that is easy to carry around (e.g., Pope et al., 2020). Posting filters preserved with 2 mL ethanol with regular postal service, might not be possible in other countries, but the ethanol can easily be replaced by a non-flammable buffer solution. Anyone who wants to try out a similar citizen science setup that involves posting filtered samples could preserve filters in non-hazardous solutions like Longmire buffer (Longmire et al., 1997) or the tissue lysis buffer that comes with the extraction kit, as these also have been shown to be able to preserve eDNA inside filters (Gargan et al., 2022; Spens et al., 2017). Although one-third (36%) of the qPCR test sets performed by students had to be discarded (Table 3), our unconventional setup (Figure 1) allowed for the simultaneous detection of eDNA from all 14 species of amphibians in multiple parallel singleplex test sets in the same qPCR, and on the same water sample the student had provided themselves. The major drawback of having students perform the laboratory qPCR setups and running the qPCR analysis is that it likely increases the proportion of non-approved controls (i.e., "failed tests"), which in this case was 36% of the performed reaction tests. This may be due to several reasons, for example, the students flipping the PCR strips the wrong way when adding reagents and template or forgetting to add a positive control template to the correct tube. A second possibility is that onset of amplification is too late for some of the assays, and that this results in late amplifying reactions are excluded due to the cycle of quantification threshold cut-off. However, this drawback of having a relatively high proportion of failed tests is mitigated by the potential for large-scale coverage of sample locations. The level of non-approved analyses could also reflect the sensitivity of the detection system itself. A more robust assay will yield a higher degree of approved analyses than a less sensitive system. It is expected, and a premise of using citizen science based methods, that untrained high school students do not have the same experience needed in order to perform qPCR analysis with the same level of laboratory accuracy as an experienced researcher or technician (Tøttrup et al., 2021), and this can lead to the inclusion of non-approved data in the results. The stochastic nature of PCR will also influence our ability to detect low levels of eDNA, which also warrants the inclusion of more replicates in both fieldwork and laboratory setup. More technical replicates per assay would have provided better monitoring of eDNA, but would also have been more costly. Students could easily have prepared two or three times as much master mix, only for the cost of additional enzyme. However, the student laboratory only had one qPCR machine available. Running the qPCR machine for a second or a third round would require the course instructor to start up these additional runs. Although the machine does not need any interaction while running, the student laboratory still needs to be tidied up and prepared for the following day. On top of this, the students would not be around to see the second round of results, and not have a discussion with the instructor. Our qPCR setup (Figure 1), where multiple species were tested on the same water sample, was also chosen to allow the students to see at least a few successful amplifications. The alternative would have been to prepare a full plate of 96 wells with standard-dilution series and only use the same assay for all 96 tubes in the PCR plate. Using the same assay for a full qPCR run for an extremely rare amphibian species could quickly become a disappointing experience for the students to witness, as some of the rare species never returned any positive detections. It would also have made it more difficult to discuss biodiversity with the students if a qPCR setup only covered a single species. Many different species targeted in the same qPCR setup make the monitoring much more interesting to the students. Furthermore, having the high school students prepare standard dilution series would also pose a risk of contaminating the laboratory with high levels of positive control DNA, which in turn would be detrimental for any future qPCR setups in the same laboratory. Another aspect to consider is that many amphibians often are more active during the night. A marine study has already pointed out that the distribution of species varies over a diurnal period, and eDNA levels will because of this vary over a day (Jensen et al., 2022), and the time point for sampling water might then be optimal for some species during the night. Any future studies that intend to use a similar monitoring setup of eDNA might want to consider whether sampling during nighttime can result in different levels of eDNA from amphibians than what can be obtained from sampling during the day. By charging students a course fee of around 14 Euro each, a class of 20 students could cover 280 Euros of the budget, with more than 100 waters samples, each representing an individual class, the project could cover more than 28,000 Euros of costs related to enzymes, reagents, pipettes and purchase of qPCR machine. This economic aspect is worth considering for future citizen science projects.

In all our qPCR setups, we ran 50 amplification cycles compared to the 55 cycles used for monitoring *Triturus cristatus* in the United Kingdom (Biggs et al., 2015). Running more amplification cycles gives a higher risk of amplifying something that stems from contamination of samples. Future studies aiming a setting up similar citizen science projects to get students involved should aim for running a maximum of 50 cycles or less, as more amplification cycles can mean a greater risk of getting false positive detections. The specific assay targeting *T. cristatus* (Thomsen, Kielgast, Iversen, Wiuf, et al., 2012) could have been compared with the performance of our own assay against *T. cristatus*. It could have been interesting to compare the performance of the two assays against *T. cristatus*, but we only realized this too late, and this will have to wait for future studies.

One of the positive outcomes of the citizen science approach is that many remote areas can be sampled in a relatively short time, and this may eventually lead to enhanced eDNA monitoring at much larger spatial scales. Because our qPCR setup requires a positive control and a negative control for each individual mix prepared for every two tests tubes of an extracted water sample (i.e., for every set of four tubes), it provides a starting point for fruitful discussions with students on how incorrect conclusions can be drawn if negative and positive controls are not included, and also gives the students a positive outcome in terms of experience in how to apply quality

control and how to work under best scientific practice. The high school students get a hands-on experience with authentic molecular laboratorial work in a professional setting and are encouraged to engage in natural science as they do field- and laboratorial work that produces data that can help to monitor rare and protected species in their local neighborhood.

Using metabarcoding for monitoring of eDNA from amphibians (e.g., Brys et al., 2021; Hawlitschek et al., 2016) might be more cost-efficient when diversity is high, as in tropical forests (Bálint et al., 2018), whereas monitoring of eDNA with specific assays (Goldberg et al., 2018; Ruso et al., 2019; Smith & Goldberg, 2020) may be considered a better approach when diversity is low (Franklin et al., 2019; Osathanunkul & Minamoto, 2021; Torresdal et al., 2017). Our choice of developing multiple individual assays was based on that we wanted the continuous running cost to be low, which is easier if assays already have been developed and validated (Harper et al., 2018), and we wanted a setup where untrained students could be brought in to do both fieldwork and final laboratorial tests, with an immediate learning outcome for the students. A metabarcoding setup would have covered all species, but such data would not allow for immediate interpretations by instructor and students. Future citizen science projects that are not required to have the participants engaged and gain an immediate learning outcome could apply metabarcoding, similar to the studies performed by Agersnap et al. (2022) and Suzuki-Ohno et al. (2023).

4.4 | Challenges of complex species and hybrids

The detection of eDNA from amphibian species has previously been attempted in Denmark, although for a more limited number of species (Thomsen, Kielgast, Iversen, Wiuf, et al., 2012). With the improved and more thoroughly tested and validated assays presented here, there is scope for a broader testing of freshwater bodies across northern Europe. Any traces of eDNA from *P. ridibundus* or *P. lessonae* can potentially stem from *P.* kl. *esculentus* as the eDNA targeted is in the mitochondrial genome, and dependent on the maternal heritage of any *P.* kl. *esculentus* detected will reflect the presence of either *P. ridibundus* or *P. lesonae*. Past attempts at determining the distribution of amphibians and reptiles in Germany have also been unable to differentiate between *P. ridibundus* and *P. lessonae* when monitoring is performed for eDNA (Hawlitschek et al., 2016). With the lowest genetic diversity for *P.* kl. *esculentus* complex in the northern part of Europe (Hoffmann et al., 2015), there is a lower risk of getting false negative detections with the assays presented here, as a low genetic diversity warrants assays that are likely to work across the span of a uniform genetic diversity. The hybridization and backcrossing of genomes among species representing *Pelophylax* impose difficulties when species are to be monitored by eDNA. The present study is only capable of detecting the eDNA from these species complexes, and not capable of inferring what morphological species it stems from. A future study could try and target a nuclear DNA region in the *Pelophylax* complex that reflects the "L" and "R" genotypes, although this might be difficult as species in the *Pelophylax* complex can be di-, tri and tetraploid (Hoffmann et al., 2015). A metabarcoding study targeting *Pelophylax* would also have a hard time differentiating between the different species of *Pelophylax* as the usual mtDNA region targeted by metabarcoding not necessarily will reflect the "L" and "R" genotypes. We recommend that future studies trying to monitor the *Pelophylax* complex perhaps start out by comparing full genomes to allow for the identification of nuclear DNA that reflects the "L" and "R" genotypes. Such an assay can be coupled with the assays we present here, to allow for monitoring of other amphibian congeners. Information about the distribution of the genetic markers targeted by the assays presented here is, however, still valuable as it provides information about the distribution of the genetic diversity in the *Pelophylax* complex, which can be used for pinpointing areas where hybridization is more likely to occur. Hence, the results on *Pelophylax* species obtained from the analysis are all reported as edible frog, which is the only species of *Pelophylax* widely distributed in southern and eastern parts of Denmark.

5 | CONCLUSION

Species-specific qPCR detection systems were designed, tested, and validated for amphibian species in northern Europe. The validation reached "level 4" according to the criteria suggested by Thalinger et al. (2021). The assays presented were validated for Denmark. However, the assays are expected to be applicable in other European regions too, when the same 14 target amphibian species constitutes all the sympatric congeners (i.e., from central Germany and northwards). The detection systems were applied in a large-scale nationwide citizen science project in which sampling and qPCR analysis was carried out by high school students. Data from the citizen science project returned the expected results when compared to the known regional distribution of the target species. Only four out of 2551 qPCR tests carried out by high school students required a professional reanalysis due to unexpected results. This emphasizes that data from citizen science may generate large amounts of valuable data when ensuring a careful curation of the results.

AUTHOR CONTRIBUTIONS

Steen Wilhelm Knudsen, Peter Rask Møller, Peter B. Mortensen, Martin Hesselsøe, Marie Rathcke Lillemark, Philip Francis Thomsen, Carsten Rahbek, and Anders P. Tøttrup conceived the ideas; Steen Wilhelm Knudsen, Peter Rask Møller, Martin Hesselsøe, Maria Rytter, Marie Rathcke Lillemark designed the study; Steen Wilhelm Knudsen, Maria Rytter, Sune Agersnap, Philip Francis Thomsen, Maria Rytter, Marie Rathcke Lillemark collected and produced experimental data; Steen Wilhelm Knudsen and Julie Koch Sheard analyzed the collected data; Steen Wilhelm Knudsen wrote the first draft of the manuscript and prepared all drawings of amphibians; Steen Wilhelm Knudsen, Peter Rask Møller, Martin Hesselsøe, Maria Rytter, Sune Agersnap, Philip

Francis Thomsen, Julie Koch Sheard finalized the manuscript. All authors contributed to the final manuscript and approved the manuscript for publication.

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CONFLICT OF INTEREST STATEMENT

Martin Hesselsøe has been employed at Amphi Consult ApS and now in NIRAS A/S. Peter B. Mortensen is employed at Eurofins Miljø A/S, and Sune Agersnap is now employed in NIRAS A/S. During the process of submitting this manuscript these companies (Amphi Consult ApS, NIRAS A/S, and Eurofins Miljø A/S) have had access to the results and exclusive rights to apply these assays for commercial purposes. Any exclusive access will be terminated through this publication which makes the data publicly accessible.

DATA AVAILABILITY STATEMENT

All data and code for data analysis can be obtained by git cloning this data repository: https://github.com/monis4567/amphibia_eDNA_in_Denmark.git. The data and code can also be obtained from this dryad data repository: https://doi.org/10.5061/dryad.w9ghx3fvc.

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SUPPORTING INFORMATION

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

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