Using eDNA to survey amphibians: Methods, applications, and challenges

Xiaoxuan Sun[1], Ningning Guo[1], Jianan Gao[1], and Nengwen Xiao[1]

[1]Chinese Research Academy of Environmental Sciences

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Abstract

In recent years, environmental DNA (eDNA) has received attention from biologists due to its sensitivity, convenience, labor and material efficiency, and lack of damage to organisms. The extensive application of eDNA has opened avenues for the monitoring and biodiversity assessment of amphibians, which are frequently small and difficult to observe in the field, in areas such as biodiversity survey assessment and detection of specific, rare and endangered, or alien invasive species. However, the accuracy of eDNA can be influenced by factors such as ambient temperature, pH, and false positives or false negatives, which makes eDNA an adjunctive tool rather than a replacement for traditional surveys. This review provides a concise overview of the eDNA method and its workflow, summarizes the differences between applying eDNA for detecting amphibians and other organisms, reviews the research progress in eDNA technology for amphibian monitoring, identifies factors influencing detection efficiency, and discusses the challenges and prospects of eDNA. It aims to serve as a reference for future research on the application of eDNA in amphibian detection.

Introduction

Amphibians are vertebrates with life cycles that crucially depend on both aquatic and terrestrial habitats. They play an important intermediate position in food chains and ecosystems, and are important indicator taxa of environmental health (Li et al., 2017). Amphibians are not only an important component of biodiversity but are also a taxonomic group that has undergone significant changes in taxonomical organization in recent years and is experiencing rapid population declines and risks of extinction at a global scale (Gao et al., 2022). China is among the countries with the richest biodiversity in the world and specifically boasts the highest diversity of amphibian species(Xu et al.,2018). Monitoring the status of this diverse fauna is often challenging. According to the *Technical Provisions on Survey and Assessment of Amphibian and Reptile Diversity in County Areas* (Ministry of Ecology and Environment Announcement No. 84, 2017), traditional methods to investigate amphibian diversity include traditional line transect methods, trap-barrier systems, methods using artificial covers or shelters, mark-recapture, call counting, and others. Traditional investigation is often accurate and intuitive, but efficiency is frequently affected by species, life stages and environmental conditions (Ruppert et al., 2022). Moreover, a high level of taxonomic knowledge is required of the traditional investigator, especially for identification of eggs and larvae (Hopkins & Freckleton, 2002; S. Zhang et al., 2020). In recent years, there has been increasing use of the environmental DNA (eDNA) method, which allows the DNA of target organisms obtained from environmental samples (e.g. water, soil, excrement, ancient sediments, etc.) to be used for detection of presence or absence of the species, and even enables inferences about their numbers and biomass (Ficetola et al., 2019; Thomsen & Willerslev, 2015). Extensive use of eDNA technology has been made for biodiversity surveillance across diverse taxa, encompassing fish (Deiner et al., 2017; Jerde et al., 2011; P. F. Thomsen, J. Kielgast, L. L. Iversen, P. R. Moller, et

al., 2012), amphibians (Goldberg et al., 2018; Pilliod et al., 2013; Strickler et al., 2015; Valentini et al., 2016), benthic fauna (Cowart et al., 2018; Laroche et al., 2018; Stoeck et al., 2018), plants (Johnson et al., 2019; Johnson et al., 2021; Kodama et al., 2022), bacteria (Laroche et al., 2018; Stoeck et al., 2018; Y. Zhang et al., 2020), and microorganisms (Handelsman, 2004; Rondon et al., 2000). Nonetheless, varied applications of eDNA technology among distinct biological cohorts elicit discernible disparities. This review provides an exposition of the applications of eDNA technology in amphibian monitoring and also discusses the multifaceted factors determining its efficacy and reliability. We also examine future research and questions that will need to be addressed for the further development of eDNA technology for amphibian population investigations.

1 Introduction of eDNA method

Due to the intensification of human activities and increased interference with the natural environment, the continuous decline of global biodiversity has become one of the major challenges in the 21st century (Butchart et al., 2010). Rapid, sensitive and accurate biodiversity survey tools are required to measure fluctuations in species diversity and abundance to inform conservation and management strategies (Kelly et al., 2014). Environmental DNA is DNA that can be extracted from environmental samples (such as soil, water or air) without prior isolation of any target organisms (Lodge et al., 2012; Taberlet et al., 2012). Environmental DNA analysis is divided into two main categories: single species identification and eDNA metabarcoding. Single species identification is mainly used for sensitive detection of rare and endangered animals and invasive non-native species in the context of environmental and species richness surveys (Wilcox et al., 2013). eDNA metabarcoding refers to analysis of eDNA with high-throughput sequencing technology, processing eDNA from water, soil and air by PCR amplification to determine the presence of species and perform biodiversity assessment (Ruppert et al., 2019). Compared with traditional survey methods, the eDNA metabarcoding technique provides outstanding species recognition. It can be used even if the investigators lack extensive abilities to classify species. Further, the use of eDNA does not require sampling approaches that are destructive or invasive to the environment or the biological community, nor does it require special equipment or particular observation times to detect those species that are difficult to observe due to small size or numbers (Deiner et al., 2017). Therefore, this technology has been widely used in the field of ecology.

2 Key aspects and challenges in eDNA monitoring of amphibians

2.1 Sample collection and DNA capture

Amphibians exhibit a bifurcated life history, comprising both aquatic and terrestrial phases. In the former, their eDNA is disseminated into the aquatic milieu via exudates from integumentary secretions, excreta, and ova deposition. In the latter, eDNA is presumed to derive from imprints generated during their terrestrial locomotion, manifesting as soil or sedimentary remnants. Consequently, a comprehensive scrutiny of amphibian eDNA mandates the simultaneous procurement of aquatic and terrestrial substrates. The sources of collecting amphibian environmental DNA samples are mainly water, soil, and other natural substrates. Some studies have demonstrated that detecting terrestrial vertebrates from soil samples is challenging and that eDNA testing is more complex under these circumstances. For example, Walker et al. cultured salamanders in a mesocosm ecosystem and found that only 1% of salamander DNA from soil samples could be successfully amplified (Walker et al., 2017). The success of applying the eDNA method for detection depends not only on the presence and concentration of eDNA in the substrate sample but also on capture potency, DNA extraction success, sample interference (such as inhibition), and assay sensitivity (Ficetola et al., 2015; MacKenzie et al., 2002; Schultz & Lance, 2015). Filtration is the standard approach for the capture of DNA from aquatic environments (Goldberg et al., 2016); generally, volumes between 15 mL and 10 L of water are sampled to trap eDNA in a filter. In contrast to fish, amphibians may exhibit comparatively modest eDNA exudation. Some judicious augmentation of filtrate volume may therefore be considered to enhance

the sensitivity of detection. Common filtration materials include nitrate cellulose (Caren S Goldberg et al., 2011), glass fiber (Jerde et al., 2011), polycarbonate (Takahara et al., 2012), nylon (Philip Francis Thomsen et al., 2012) and polyether sulfone (Renshaw et al., 2015). Chen et al. evaluated the ability of 12 different types of adsorption materials and filters to capture eDNA in both laboratory and field experiments using quantitative PCR (qPCR) analysis. They found that the ability of a glass fiber filter (GF) to capture eDNA was superior to other materials, and that the amount of eDNA captured increased linearly over 72 hours (Chen et al., 2022).

It has been demonstrated that the period of detectability of eDNA in water samples is limited to between 1 day and 8 weeks (Dejean et al., 2011; Pilliod et al., 2014; Philip Francis Thomsen et al., 2012; P. F. Thomsen, J. Kielgast, L. L. Iversen, C. Wiuf, et al., 2012), and therefore false negative results may be obtained if there is a delay in the collection of water samples relative to the time of species presence. In addition, temperature alterations caused by seasonal changes will also affect the content of eDNA in samples. For example, Wei et al. studied the variation over time of eDNA in sediments over periods of one year, one month and one tidal period, and found that eDNA content was higher in the warm season and lower in the cold season (Wei et al., 2019). Similarly, the concentration and distribution of eDNA can be influenced by spatial factors such as spatial dissipation of eDNA and the flow of the water column. In still water, the eDNA of species decreases significantly within a radius of 5–10 m from the eDNA point source (Brys et al., 2021), while in flowing waters, eDNA may move with the current to new locations hundreds of meters away (Pilliod et al., 2013), leading to false negatives at the original point as well as false positives at the new location.

Therefore, biases in results due to temporal and spatial differences need to be taken into account when designing eDNA experiments, and these factors also need to be considered when conclusions are drawn about the range of species present. For example, conclusions drawn from water samples taken along one side of a river are not directly representative of the whole river.

2.2 DNA extraction

During amphibian DNA extraction, pollution should be avoided and the facilities and equipment employed should be disinfected. Standard autoclave methods are not sufficient to destroy nucleic acid molecules(Unnithan et al., 2014). Among common stain removal methods, sodium hypochlorite solvent is the most effective for removing DNA and PCR products (Champlot et al., 2010; Prince & Andrus, 1992). The choice of eDNA extraction method after filtration can greatly affect the detection of eDNA. Deiner et al. compared the effectivity of six different combinations of sample collection and DNA extraction methods in biodiversity monitoring in water ecosystems. The results showed that different environmental DNA collection and extraction methods had a significant effect on DNA yield and the number of sequences obtained by high-throughput sequencing techniques (Deiner et al., 2015).

Common DNA extraction methods include liquid phase separation and commercial DNA extraction kits. Trials have shown that liquid phase separation methods can in some cases outperform kits in terms of extraction efficiency. Turner et al. showed that a cetyltrimethylammonium bromide (CTAB) method using polycarbonate orbital filters collected more eDNA than a PowerWater DNA isolation kit using GF membranes (1.5 μm pore size, 934-AH grade) (Turner et al., 2014). It has also been shown that the phenol-chloroform-isoamyl alcohol extraction (PCI) method using glass and nitrocellulose membranes extracted more DNA than the DNeasy kit (Deiner et al., 2015; Renshaw et al., 2015). However, liquid phase separation methods are difficult to apply widely due to the utilization of toxic substances such as phenol and chloroform (Tsuji et al., 2019).

2.3 Primer selection and PCR amplification

Species-specific detection is carried out the basis of PCR techniques that use specific primers to amplify and detect short segments of DNA (typically 80–200 bp) of the target species (Bohmann et al., 2014). eDNA was first applied to large organisms using specific primers that amplified only one species during

PCR (Jerde et al., 2011; P. F. Thomsen, J. Kielgast, L. L. Iversen, C. Wiuf, et al., 2012). Advances in sequencing technology have made it possible to assess the community composition using universal primers that identify multiple species in a sample; universal primers are required to amplify all species in a target taxonomic unit with a minimal bias to avoid preferential amplification of some species with mismatched primer regions (Ficetola et al., 2010). The mitochondrial genes *12S* and *Cytochrome b* and the mitochondrion's D-loop region are commonly used in amphibian studies, and the *12S* region is often used for metabarcoding eDNA analysis (Table 1) (Li et al., 2021; Wynne E Moss et al., 2022; Sasso et al., 2017). For example, Valentini et al. designed a pair of primers specific for amphibians and scleractinians (batra_F, 5'-ACACCGCCCGTCACCCT-3,' batra_R, 5'-GTAYACTTACCATGTTACGACTT-3') and a human DNA blocking primer (batra_blk, 5'- TCACCCTCCTCAAGTATACTTCA-AAGGCA-SPC3I-3'); this primer pair can amplify up to 99% of amphibian species (Valentini et al., 2016). In a previous study, we employed GF filtration membranes in conjunction with the "batra" primers to investigate the amphibian diversity within China's Chao Lake(Unpublished data). This approach not only facilitated the successful amplification of all species identified through traditional sampling methods but also unveiled two species that had previously eluded detection in historical records and conventional surveys. These outcomes substantiate the efficacy of the 'batra' primers for detecting Chinese amphibians.

Because it is impossible to pinpoint organisms directly based on eDNA molecules exuded into water, nor to track the movement of specific eDNA molecules in water, the environmental samples collected often contain mixed DNA from many different organisms. When amplifying environmental samples, the specific primers chosen often play an important role in the successful amplification of the target species. Unsuitable primers have the potential to generate false positives, which can lead to overestimates of the presence or abundance of endangered species, or to false negatives, which can lead to insufficient monitoring of invasive species. In his study, Kelly pointed out that using a constant number of PCR cycles, different primers showed different biodiversity structures for the same simulated community (Kelly et al., 2019), which directly led to different analysis results. Wilcox tested the factors affecting the specificity and sensitivity of eDNA detection, and found that specificity was most affected by the mismatch of base pairs in the primers, which was much more prevalent than in the probe (Wilcox et al., 2013). Hence, it is evident that the meticulous selection of appropriate primers is of paramount importance.

$$Cq = (\mathrm{Cq}_{\mathrm{samples}} - \mathrm{Cq}_{\mathrm{positive\ control}})$$

It has been demonstrated that diluting the sample alleviates the inhibition, but likewise dilutes the target DNA. Especially when the target DNA is present at very low concentrations, this may lead to failure of the assay (Goldberg et al., 2013; Anna M McKee et al., 2015; Strand et al., 2011; Tsai & Olson, 1992). McKee et al. examined this using a PCR inhibitor removal kit and found some alleviation of inhibition but also potential loss of target DNA (Anna M McKee et al., 2015).

2.4 Sequencing and data analysis

The PCR results are recorded as negative or positive. A negative result indicates that eDNA of the target species is not present in the environmental sample, while a positive result indicates its presence. This result is initially determined by GEP after primer amplification and then further verified by sequence comparison. In qPCR, a fluorescence threshold is set using the positive control as a benchmark; the sample is labelled positive when the amplification exceeds the fluorescence threshold, and negative when it does not (Venkatesan & Bashir, 2011). Metabarcoding uses universal PCR primers to amplify DNA in the environment. A large amount of data containing taxonomic information on genes can be obtained after high-throughput sequencing, which requires sequence comparison and species annotation (including noise removal and assignment of operational taxonomic units), clustering, and assignment of species annotation.

A complete and high quality reference database is the basis for the accuracy of amphibian surveys using eDNA technology (Alexander et al., 2020; West et al., 2022). This can be constructed by consulting established

publicly available databases (e.g. NCBI: https://www.ncbi.nlm.nih.gov/) or by building custom versions. For example, Valentini et al. built a reference database containing 53 species of amphibians when using eDNA method to investigate amphibian diversity (Valentini et al., 2016). The accuracy of self-built databases is likely to be higher than that of public databases due to the significant geographic variation in amphibian fauna and the lack of assurance of the quality of DNA barcoding in publicly available databases.

3 Progress of domestic and international eDNA-based surveys of amphibians

3.1 Species diversity surveys

With the signing of the Convention on Biological Diversity (CBD) and the 15th Conference of the Parties (COP15) to the United Nations Framework Convention on Climate Change (UNFCCC), global biodiversity has received wide attention. eDNA technology combined with Next-generation sequencing offers a more sensitive and efficient method for biodiversity surveys. Svenningsen et al. used simultaneously traditional survey methods and eDNA metabarcoding to detect amphibian diversity in Danish lakes and ponds, and evaluated the detection efficiency of the two methods from the perspectives of species richness, the average number of species detected at various points, and frequency of detection. They found that both eDNA metabarcoding and traditional surveys yielded a large number of observations unique to the respective method, suggesting that eDNA metabarcoding should be combined with traditional methods as a complement to enhance determination of the total composition of species (Svenningsen et al., 2022).

eDNA detection of amphibians has yielded good results in temperate regions (Deiner et al., 2017; Thomsen & Willerslev, 2015; Yates et al., 2019). However, it has been demonstrated that high temperatures and strong UV light favor the growth of microorganisms and promote the degradation of DNA. To validate eDNA metabarcoding surveys of amphibian diversity in the tropics, Sasso et al. analyzed four streams in the Atlantic Forest of southeastern Brazil during a five-year period of traditional field surveys (using visual and acoustic methods) as well as short-term sampling (4 days) of eDNA. A total of 10 species of aquatic amphibians were detected over the 5-year period of the traditional survey, while nine species were detected by eDNA metabarcoding, including one species that had only been detected once during the 5-year traditional survey. There was a high degree of similarity in stream community composition between the results of the two methods (Sasso et al., 2017). Li et al. used eDNA metabarcoding to assess amphibian diversity at 288 sites in 18 regions of Hainan Island and found 15 species, including a number difficult to detect with traditional monitoring methods (Li et al., 2021). These studies demonstrate the effectiveness of the eDNA metabarcoding technique in detecting amphibians in the tropics.

3.2 Detection of invasive species

Alien invasive species are one of the main causes of global biodiversity loss and homogenization (Ehrenfeld, 2010; Ficetola et al., 2007; Pysek & Richardson, 2010; Vitousek et al., 1997). Invasive species may disrupt local ecological balance and disturb the ecosystem, with substantial impacts on the variety and abundance of native species (Ficetola et al., 2008). The early presence of invasive species is difficult to detect in a timely manner using traditional methods, while eDNA technology allows for rapid and sensitive monitoring of invasive organisms. Invasive amphibians were the first vertebrates for which species distribution was successfully assessed by extracting DNA from water samples (Ficetola et al., 2008). Specific primers were used to expand short mitochondrial DNA sequences to trace the presence of invasive bullfrogs (*Rana catesbeiana*) in controlled environments and natural wetlands. Similarly, Dejean et al. assessed the distribution of this species in ponds in western France using traditional monitoring (visual and acoustic surveys) and eDNA. Bullfrogs were found in only 14% of sites in the traditional survey, while the eDNA survey detected a much higher presence (78%), demonstrating substantial effectiveness and sensitivity (Dejean et al., 2012).

3.3 Detection of rare and endangered species

eDNA has great potential for detecting rare and endangered species. Pierson et al. investigated the occurrence of the tiny, rare, multi-toothed salamander *Urspelerpes brucei* by using both the litter bag survey and eDNA methods. The results showed that the detection probability of the eDNA survey was 0.788, much higher than that of the litter bag survey (0.048). The eDNA method also imposes weaker disturbances on aquatic habitats and has lower costs than the litter bag method (Pierson et al., 2016). Goldberg et al. designed specific primers to detect two rare species in the United States,*Ascaphus montanus* and *Dicamptodon aterrimus* . In the experiment, water samples were collected from five rivers with different densities of the target species, and PCR amplification was performed to detect whether the species' DNA existed in the samples.*Dicamptodon* was detected in all water samples and *Ascaphus*in four of the five rivers, proving the effectiveness of eDNA detection for surveys of these rare species (Caren S Goldberg et al., 2011).

McKee et al. studied the species *Ambystoma cingulatum* ,*Ambystoma bishopi* , *Notophthalmus perstriatus* and*Lithobates capito* in the southeastern longleaf pine region of the United States using both eDNA and traditional methods (mainly dip-netting, trapping, and visualization). The eDNA method succeeded in detecting the target species at six sites where traditional methods did not show a presence, again indicating that eDNA can be used as a supplement to traditional methods for investigating endangered species (A. M. McKee et al., 2015). Voros et al. used eDNA to detect the presence of the rare cave-dwelling amphibian *Proteus anguinus* in all of 15 sampled caves in Croatia, five of which constituted first records for the species (Voros et al., 2017). Lopes et al. analyzed water samples from the Atlantic Coast Forest and six mountainous areas of the adjacent Cerrado grasslands in Brazil for traces of DNA of rare and endangered amphibian species. They successfully detected four declining species (*Hylodes ornatus* , *Hylodes regius* ,*Crossodactylus timbuhy* and *Vitreorana eurygnatha*), two species considered locally extirpated (*Phasmahyla exilis* and*Phasmahyla guttata*), and a species not seen since 1968 (*Megaelosia bocainensis*) (Lopes et al., 2021). These studies successfully confirmed the presence of species not detected by traditional methods, and underline the effectiveness of the eDNA method for biodiversity monitoring at low population densities.

3.4 Species abundance and biomass assessment

eDNA allows for the assessment of the relative abundance and biomass of faunal communities. In 2019, Kelly et al. analyzed eDNA macro-barcodes of three simulated biomes with different biomass ratios. The effects of these ratios on biodiversity estimates were analyzed by assuming that the amount of DNA collected in the environment was proportional to abundance of individuals in the water, and that the DNA shedding rate and amplification efficiency of certain primers of isotaxa was constant. The analysis results showed that in the case of high amplification efficiency, such as primer adaptation, the ratio index of eDNA macro-barcode readings was highly indicative of trends in group biomass (Kelly et al., 2019). This result has been replicated in amphibian studies. For example, Thomsen et al. investigated six different species including two amphibians (*Pelobates fuscus* and *Triturus cristatus*) using eDNA and qPCR methods, finding that the eDNA concentrations of these two species in water samples correlated with density and biomass (P. F. Thomsen, J. Kielgast, L. L. Iversen, C. Wiuf, et al., 2012). Pilliod et al. used eDNA and traditional methods to assess the diversity and abundance of two amphibian species,*Ascaphus montanus* and *Dicamptodon aterrimus* , in 13 streams in central Idaho, USA. They reported that eDNA reading proportion was positively correlated with density and biomass as measured by traditional methods, and showed that the accuracy of eDNA-based abundance assessments increased with the amount of eDNA in water and the number of replicate samples collected (Pilliod et al., 2014). Everts et al. used droplet digital PCR (ddPCR) analysis of eDNA to detect the abundance of bullfrogs in the United States, and found that eDNA concentration increased significantly with increasing abundance of bullfrog eggs and tadpoles (Teun Everts et al., 2021).

3.5 Reconstruction of ancient ecosystems

eDNA for amphibian surveys is mainly used to detect the presence of target species in current communities. However, it has been shown that since DNA molecules can exist in the environment for a long time, ancient ecosystems can be assessed by extracting remnant DNA molecules from environmental sources. Ficetola and Taberlet detected eDNA of several amphibian species (*Bufo bufo* , *Rana temporaria* and*Ichthyosaura alpestris*) in 1,000-year-old sediments from a lake in the French Alps. This suggests that eDNA stored in lake sediments can be used to understand changes in species distribution over time. In addition, it can be combined with other paleoecological data to understand species' responses to environmental changes (such as habitat alterations, climate change, and the introduction of alien species) (Giguet-Covex et al., 2019).

3.6 Amphibian disease surveillance

In addition to factors such as habitat degradation and environmental pollution, viral infections have also emerged as a significant contributor to the decline of amphibian populations (Rachowicz et al., 2006). According to Scheele et al., the amphibian chytrid fungus (*Batrachochytrium dendrobatidis* ; Bd) has led to the decline of at least 501 amphibian species worldwide, with 90 species having gone extinct, including the Darwin's frog (*Rhinoderma darwinii*) (Scheele et al., 2019). In recent years, eDNA methods have demonstrated remarkable capabilities for detecting Bd due to their convenience, sensitivity, and non-invasive nature.

Researchers like Stephen et al. have successfully determined the presence and quantity of chytrid pathogens by collecting skin swabs from amphibian hosts, extracting DNA from these swabs, and employing qPCR techniques (Boyle et al., 2007). Beyond skin swab collection, pathogenic DNA can also be detected from environmental samples. Walker et al., for instance, successfully detected the presence of the Bd in small-volume (<1 L) water samples by filtering and utilizing qPCR (Walker et al., 2007). Similarly, Julie et al. employed filtration to capture zoospores from water samples and subsequently detected pathogen DNA from filtered particles at concentrations as low as 0.06 zoospores per water sample, highlighting the high analytical sensitivity of the eDNA method (Kirshtein et al., 2007).

The potential of eDNA technology for early detection of Bd in the environment is noteworthy. Colleen et al. collected eDNA from filtered water samples and detected Bd at three sites, all of which a month later turned out to experience Bd-induced mortality, while Bd was not detected in sites without later mortality (Kamoroff & Goldberg, 2017). This underscores the potential of eDNA technology for early detection of Bd presence.

4 Influential factors of eDNA detection

Lindahl showed that the detection and quantification of DNA in specific freshwater animal species depends on DNA release and degradation rates. eDNA release is related to population density and organism size, and DNA degradation is determined by a simple relationship between microbial or enzymatic attack and spontaneous chemical reactions such as hydrolysis and oxidation (Lindahl, 1993). Conceptually, three processes determine the detection of DNA in environmental samples: production, dissipation and degradation.

4.1 Production of eDNA

For aquatic eDNA, production, the rate at which DNA is released in water, is a highly variable function, influenced by population density and species-specific characteristics, such as individual size and metabolic rate (Strickler et al., 2015). Thomsen conducted Pearson correlation analysis of population size and the mean number of DNA molecules in ponds for two amphibian species (*Pelobates fuscus*and *Triturus cristatus*) and found that DNA release increased with population density and animal size (P. F. Thomsen, J. Kielgast, L. L. Iversen, C. Wiuf, et al., 2012). In order to examine the effect of different growth stages of amphibians

on the efficiency of eDNA detection, Everts et al. compared the eDNA emission rates of tadpoles and juveniles of American bullfrogs (*Rana catesbeiana*) and found that the average individual eDNA emission rate of tadpoles was not significantly different from that of juveniles, while the average emission rate per unit biomass of tadpoles was significantly lower than that of juveniles (Teun Everts et al., 2021).

4.2 Dissipation of eDNA

The dissipation pattern of eDNA was shown to vary between species and experimental conditions. In order to obtain more accurate results for amphibian detection by eDNA methods, Brys et al. estimated the temporal and spatial dissipation of eDNA by placing a group of different fish and amphibians in a cage on one side of a pond (thereby creating eDNA emission sites) and assessing the dissipation of eDNA with distance by collecting water samples at different distances from the cage. Detection rate of the locally released eDNA and the standardized index of relative eDNA abundance of the cage species decreased significantly with distance within a radius of 5–10 m from the cage. After removing the cage community for one week, no DNA of any species could be detected in the study system, indicating that the eDNA had degraded below the detection threshold within that period (Brys et al., 2021). Dejean et al.'s study found that eDNA from American bullfrog (*Rana catesbeiana*) tadpoles was able to persist for 25 days under a laboratory microscope (Dejean et al., 2011). Thomsen et al. found that in experiments conducted in outdoor ponds, eDNA from larvae of the amphibians*Pelobates fuscus* and *Triturus cristatus* could be detected for 7–14 days (P. F. Thomsen, J. Kielgast, L. L. Iversen, C. Wiuf, et al., 2012).

4.3 Degradation of eDNA

Studies have shown that degradation of DNA in water is one of the main factors that reduce detectability (Barnes et al., 2014; Dejean et al., 2012; Pilliod et al., 2014). An understanding of the relevant environmental factors is therefore essential for controlling degradation rate and optimizing sampling strategy when using eDNA methods. DNA in water is broken down by chemical hydrolysis, primarily by exposure to acids or by enzymatic action. Microbial activities in water directly promote enzymatic hydrolysis by producing exogenous nucleases, which break down DNA into its components (Lindahl, 1993).

High temperatures accelerate the degradation of DNA (Corinaldesi et al., 2008; Okabe & Shimazu, 2007). When temperatures are very high (>50°C), direct denaturation can occur; however, most of the effects of high temperatures on DNA degradation arise indirectly, as moderately high temperatures stimulate microbial metabolism and exonuclease activity (Corinaldesi et al., 2008; Fu et al., 2012; Hofreiter et al., 2001; Pote et al., 2009; Zhu, 2006). Strong ultraviolet radiation can also enhance DNA degradation. This particularly applies to ultraviolet B (UV-B) light, as UV-B wavelengths are the most harmful to aquatic organisms among wavelengths most likely to occur in terrestrial sunlight (Diffey, 2002), causing photochemical damage to DNA and thus inhibiting DNA amplification (Hader et al., 2003; Ravanat et al., 2001). Acidic conditions catalyze the hydrolysis process that degrades DNA (Alaeddini et al., 2010; Lindahl, 1993), and thus eDNA is present for longer in samples with neutral or slightly alkaline pH (Lindahl, 1993).

A study by Pilliod et al. clearly quantified the influence of specific environmental factors on eDNA degradation. eDNA of *Dicamptodon aterrimus* can persist for 8 days in an outdoor container under ambient light and temperature conditions, at least 11 days under ambient temperature and low light conditions, and for at least 18 days under refrigeration without light (Pilliod et al., 2014). Similarly, to quantify the independent and interactive effects of environmental factors on eDNA persistence, Strickler et al. measured eDNA degradation rates at different levels of UV-B, pH, and temperature in a laboratory setting. The results showed that temperature had a strong effect on the amount of eDNA detected over time; the degradation rate at 5°C was significantly lower than that at 20°C and 35°C, indicating slower microbial growth at low temperatures, which may delay the microbial degradation process of eDNA. There was no significant difference in degradation rate between pH 4 and pH 7, but rates at both pH levels were higher than that at pH 10. There was no significant difference in the degradation rate of UV-B at 2, 25 and 50 KJ/m^2/day, however,

the degradation rate was faster at higher UV-B levels when the level was between 5 and 25 KJ/m^2/day and the amount of residual eDNA was lower than 5%. This indicates that there is a nonlinear correlation between UV-B intensity and eDNA degradation (Strickler et al., 2015). Since geographical features such as latitude and altitude are the main determining factors of UV-B radiation (Godar, 2005), radiation effect on eDNA degradation is more significant in aquatic environments at high altitudes or near the equator. Although studies have shown that higher acidity (pH 4) accelerates the degradation of eDNA, McKee et al. successfully detected four endangered amphibian species (*Ambystoma cingulatum* , *Ambystoma bishopi* , *Notophthalmus perstriatus* and *Lithobates capito*) in natural acidic wetlands (pH close to 4) of longleaf pine in the southeastern United States (A. M. McKee et al., 2015). Goldberg et al. also detected the endangered species *Ambystoma bishopi* and*Pseudacris ornata* at the Eglin Air Force Base and demonstrated that a pH value as low as 4 would not degrade eDNA to an undetectable state (Goldberg et al., 2018).

5 Prospects

The implementation of eDNA methodologies for amphibian surveillance encompasses a sequence of five primary stages: sample acquisition, DNA extraction, PCR amplification, sequencing, and data analysis (Figure 1). It is evident from this review that the heterogeneous array of research objectives often engenders considerable divergence within the procedural framework. Noteworthy variables encompass the volumetric scale of aquatic samples, ranging from 15 mL to 10 L, the selection of filter pore sizes spanning the interval from 0.22 μm to 1 μm, and the deliberate designation of genetic loci such as 12S, 16S, and Cytochrome b, each of which shows pronounced heterogeneity. This intricate landscape precludes the facile derivation of a universally 'omnipotent' experimental protocol. It should be noted that attempts have been made to propagate standardized methodologies for eDNA techniques (Bruce et al., 2021; De Brauwer et al., 2022; Minamoto et al., 2021), however these so far have not encountered widespread acceptance or practical implementation (Takahashi et al., 2023).

The multifaceted paradigm underlying eDNA application, as discussed in section 2 of this review, illustrates the causes for the limited applicability of 'standardization guidelines' across diverse contexts. The unique physicochemical characteristics of different environments make it necessary to carry out iterative adjustments to the experimental approach blueprint and the methodology, as suitable to varied research goals (Pawlowski, Apothéloz-Perret-Gentil, et al., 2020; Pawlowski, Apothéloz-Perret-Gentil, et al., 2020; Taberlet et al., 2018; Takahashi et al., 2023).

Because spatial and temporal differences have a large and unavoidable effect on eDNA capture, it may be possible to improve the accuracy of eDNA methods for investigating species diversity or richness of plants and animals by combining them with distribution or occupancy models.

A high-quality and accurate reference database is required for eDNA metabarcoding, and the information base must be sufficient to cover all species in that experimental region. Errors or gaps in the database will lead to a decrease in the accuracy of the findings (Abad et al., 2016; Šigut et al., 2017; Yang et al., 2017). Continued enrichment and improvement of DNA barcode databases in the future are therefore desirable to improve their accuracy and credibility.

Despite the convenience, accuracy and low cost of the eDNA method, it has many disadvantages compared to traditional surveys, such as the inability to directly observe the life stages and disease conditions of surveyed organisms or to measure organism indicators. eDNA metabarcoding cannot completely replace traditional survey methods, and choice of survey methodology should be weighed against the advantages and limitations of both types of approach, as well as specific research objectives. Complementary use is most likely to achieve the most desirable research results.

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Tables

Table1. Primers used in eDNA amphibian surveys

Aim

Research Area

Mitochondrial Genome Region

Primer Name

Forward Primer(5'-3')

Reverse Primer(5'-3')

Length

Reference

Threatened Species (*Latonia nigriventer*)

Northern Israel

12S

/

(5'- GAACTACGAGCCTCAGCTTAAA-3') (5'- GGCAAGAAGTGGTGAGGTTA-3')

110 bp

(Renan et al., 2017)

Threatened Species (*Ambystoma cingulatum*, *Ambystoma bishopi*)

Southeastern United States

D-loop

Amcin F Amcin R Amcin P

(5'-GGCCCGTCAACTTTCCTCTAA-3') (5'-TGGTCCAGGTAAATCAATTGCA-3') (6FAM-TACGGTAATATGTCTGGTACTAC-MGBNFQ)

132 bp

(Anna M McKee et al., 2015)

Threatened Species (*Notophthalmus perstriatus*)

NADH Dehydrogenase Subunit 1 (ND1)

Noper F Noper R Noper P

(5'-CCGAGCCCCCTTCGAT-3') (5'-CTTCCTGCATATTCTACGTTAAATCCT-3') (6FAM-TAACAGAAGGTGAATCTG-MGBNFQ)

71 bp

Threatened Species (*Lithobates capito*)

NADH Dehydrogenase Subunit 2 (ND2)

Licap F Licap R Licap P

(5'-CGGCACTACAGTCACCCTATC-3') (5'-TCGGGATAATGGCGAGGGTAT-3') (CAL Fluor Red610-TYCATTGACTCTTAGCCTGAGTAGGCYTA-BHQ-2)

82 bp

Threatened Species (*Phytotriades auratus*)

Trinidad, America

Cytb

GTF-F GTF-R

(5'-CCCCTT ACATCGGCACTGAC-3') (5'-CTCCAAGGATGTTTGGGGTGA-3')

390bp

(Brozio et al., 2017)

Threatened Species (*Babina subaspera*)

Amami-Oshima Island,Japan

ND5

BsubND5-F BsubND5-R

(5'-TCACTCAATGCCCGCTTATATA-3') (5'-GTACTCTAAAACCAATTTAGTA-3')

90 bp

(Takahara et al., 2020)

Threatened Species (*Odorrana splendida*)

ND5

OsplND5-F OsplND5-R

(5'-GCTGAGAAGGCGTAGGATTT-3') (5'-ACATCTCATGTCGAACATTGC-3')

96 bp

Threatened Species (*Odorrana amamiensis*)

ND5

OamaND5-F OamaND5-R

(5'-TCGGCCCAGGCCCTTTTGAGCA-3') (5'-TGATTGTGTTCCCACTA-3')

116 bp

Rare Species (*Proteus anguinus*)

Dinaric Karst, Croatia

D-loop

Paf8 Par8

(5'-GTGGCATATAAATCTATGTC-3') (5'-TRTTATTCGTTTTCTAGG-3')

64 bp

(Vörös et al., 2017)

Rare Species (*Phytotriades auratus*)

West Indies

Cytb

/

(5'- GCGGATTCTCTGTCGACAATG -3') (5'- TGCTCCTGCAATAAGAAATGGA -3')

80bp

(Torresdal et al., 2017)

Secretive Species (*Ascaphus montanus*)

Northwest United States

Cytb

Asmo F Asmo R

(5'-CGTCAACTATGGCTGGCTAA-3') (5'-TCGGCCAATGTGAAGATAAA-3')

78 bp

(C. S. Goldberg et al., 2011)

Secretive Species (*Dicamptodon aterrimus*)

Dicamp F Dicamp R

(5'-TCTGCATCTTYCTACATATYGGAC-3') (5'-ATCACYCCGACKTTTCAGGT-3')

85 bp

Alien Invasive Species Quantitative Detection (*Lithobates catesbeianus*)

Western European

Cytb

BullfrogF BullfrogR

(5'-TTTTCACTTCATCCTCCCGTTT-3') (5'-GGGTTGGATGAGCCAGTTTG-3')

84 bp

(T. Everts et al., 2021)

Alien Invasive Species Quantitative Detection (*Lithobates catesbeianus*)

Western European Beijing, China

16S

qLC16S Forward qLC16S Reverse

(5'-GCAGAGATAACCTCTCGT-3') (5'-GTCCCATAGGACTGTTCT-3')

120 bp

(T. Everts et al., 2021) (Lin et al., 2019)

Alien Invasive Species (*Lithobates catesbeianus*)

Southwest of France

Cytb

/

(5'-TGCCAACGGAGCATCATTC-3') (5'-ATAAAGGTAGGAGCCGTAGT-3')

79 bp

(Dejean et al., 2012)

Quantitative Detection

Linkebeek, Belgium

12S

12S_F1 12S_R1

(5'-ACTGGGATTAGATACCCC-3') (5'-TAGAACAGGCTCCTCTAG-3')

142 bp

(Brys et al., 2021)

Quantitative Detection (*Ascaphus montanus*)

Idaho, United States

Cytb

qASMOF qASMOR

(5'-ACGTCAACTATGGCTGGCTAATC-3') (5'-GTCCTCGGCCAATGTGAAGA-3')

within 200 bp

(Pilliod et al., 2013)

Quantitative Detection (*Dicamptodon aterrimus*)

qDIATF qDIATR

(5'-AAACTTTACACCAGCAAACCCATT-3') (5'-CGGAGAATAGCGTATGCAAAAA-3')

Species-specific detection (*Hynobius kimurae*)

Kyoto Prefecture, Japan

12S

Hynobius_12S_F1 Hynobius_12S_R1 Hida_12S_P

(5'-TTAATAAAAACGGCCTAAAGCGTG-3') (5'-TCAATTATAGAACAGGCTCCTCTAGGG-3') ([FAM]-CCTTAAACTTTGGAGCCTACCCGCCTG-[TAMRA])

24˜27 bp

(Jo et al., 2020)

Biodiversity survey

Hainan Island,China

12S

batra_F batra_R batra_blk

(5'-ACACCGCCCGTCACCCT-3') (5'-GTAYACTTATGTTACTT-3') (5'-TCACCCTCCTCAAGTATACTTCAAAGGCA-SPC3I-3')

150 bp

(Li et al., 2021)

Biodiversity survey

France

12S

batra_F batra_R batra_blk

(5'-ACACCGCCCGTCACCCT-3') (5'-GTAYACTTATGTTACTT-3') (5'-TCACCCTCCTCAAGTATACTTCAAAGGCA-SPC3I-3')

20˜60 bp

(Valentini et al., 2016)

Biodiversity survey

California, United States

12S

12S-V5-F 12S-V5-R

(5'-ACTGGGATTAGATACCCC-3') (5'-TAGAACAGGCTCCTCTAG-3')

106 bp

(W. E. Moss et al., 2022)

Biodiversity survey

Brazilian Atlantic Forest

12S

batra_F batra_R batra_blk

(5'-ACACCGCCCGTCACCCT-3') (5'-GTAYACTTATGTTACTT-3') (5'-TCACCCTCCTCAAGTATACTTCAAAGGCA-SPC3I-3')

52 bp

(Sasso et al., 2017)

Figures

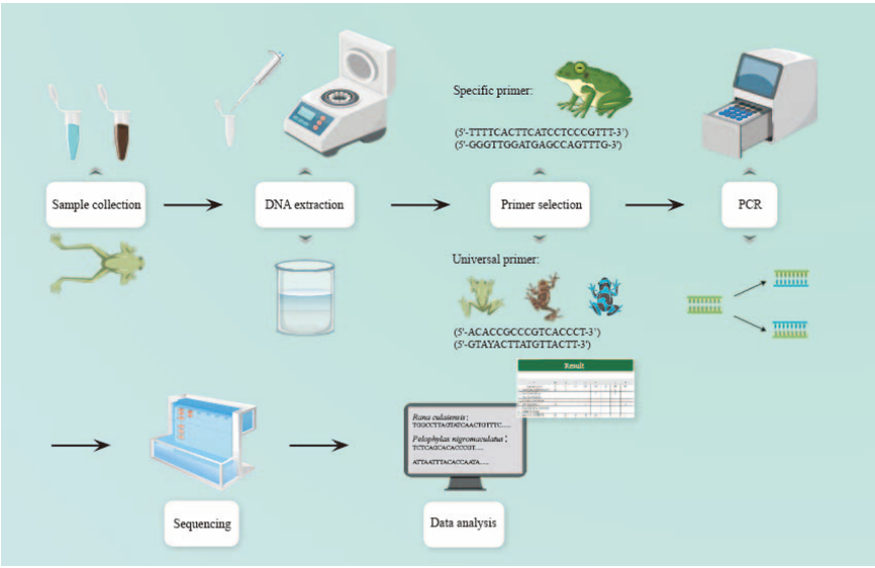

Figure 1. Flow chart of eDNA detection of amphibians