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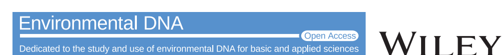

Validation of an eDNA-based workflow for monitoring inter- and intra-specific CytB haplotype diversity of alpine amphibians

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Abstract

Environmental DNA (eDNA) analysis is a promising tool for monitoring wild animal populations and, more recently, their genetic variability. In this study, we used the mitochondrial Cytochrome B gene to develop and apply new eDNA metabarcoding assays targeting amphibian families and genera in order to estimate both inter- and intraspecific genetic diversity. We designed and tested seven new primer pairs (a) in silico against an amphibian reference database based on the target genera; (b) in vitro on tissue samples of the target species; and (c) in situ on water samples from 38 wetlands in the Province of Trento (Italy). Overall, most target species were amplified successfully, although some markers also amplified non-target amphibian species. In addition, to complete the workflow, we compared the performance of three different bioinformatic pipelines (namely, MICCA with VSEARCH, and OBITools using ecotag or metabinkit), in retrieving reads and exact sequence variants from the metabarcoding datasets. Overall, the MICCA based pipeline retrieved more reads, but less putative haplotypes of amphibians. After comparing these sequences with previously known haplotypes from tissue-based studies, when the aim is to both decrease the probability of detecting false haplotypes and retrieve the highest number of reads, we suggest using MICCA+VSEARCH, unless a direct comparison with tissue-based genetic data is possible.

KEYWORDS
amphibians, bioinformatics, eDNA, environmental DNA, freshwater, genetic diversity, metabarcoding, pipelines

1 | INTRODUCTION

With 41% of known species assessed as 'threatened' by the IUCN (IUCN, 2022), amphibians are currently the most endangered vertebrate taxon on the planet (Hoffmann et al., 2010). Precise estimates of the abundances and distributions of amphibians are often difficult and time-consuming to obtain, due to the cryptic lifestyle and small size of adults, but also to the challenges of detecting and

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correctly identifying their early life stages in secluded and season-dependent aquatic environments (Barata et al., 2017; Ficetola et al., 2019).

In the last 15 years, environmental DNA (eDNA) approaches have been increasingly used for accurate and cost-efficient detection of wetland-dependent amphibian species (see Ficetola et al., 2019, and references therein). Most studies have focused on monitoring single species, showing the potential for eDNA surveys to increase the speed and efficiency of amphibian detection in comparison to traditional observational methods (Fediajevaite et al., 2021; Moss et al., 2022; Zanovello et al., 2023). The eDNA metabarcoding approach is being applied as a cost-effective method for the assessment of species composition of amphibian communities, although its performance compared to traditional approaches is still debated (Cristescu & Hebert, 2018; Ficetola et al., 2019; Moss et al., 2022; Svenningsen et al., 2022).

More recently, the potential of eDNA to estimate the genetic diversity within-species has also been investigated (Andres et al., 2023; Elbrecht et al., 2018; Sigsgaard et al., 2020 and references therein), motivated by the crucial role that genetic diversity plays in the local adaptation and persistence of natural populations in the face of environmental and climatic changes (Hoban et al., 2013; Höglund, 2009). Amphibian species are particularly vulnerable to genetic erosion due to their small effective population sizes and low dispersal rates (Allentoft & O'Brien, 2010). In addition, many amphibian species in the Mediterranean area reproduce in small temporary ponds and streams, which are highly fragmented habitats where land use changes and frequent droughts (Nadin, 2008) favor isolation and reduce gene flow and colonization potential.

Given the vulnerabilities listed above, effective monitoring protocols that aim at protecting wild amphibian species should also include the possibility of gathering population data on genetic diversity and gene flow. However, only a few articles have been published thus far on the application of eDNA approaches for the study of amphibian mitochondrial lineages (Gorički et al., 2017; Wang et al., 2022), and, to our knowledge, only one has aimed at estimating population-level genetic diversity (Zanovello et al., 2023).

Here, we (1) developed a family- to genus-specific eDNA metabarcoding assays to estimate both interspecific and intraspecific genetic diversity of Alpine amphibians, and (2) evaluated the efficiency of three different bioinformatic pipelines in filtering non-target DNA from the dataset while retaining amphibian sequence variants.

Most primers developed for eDNA can be divided into two categories: single-species targeting primers, in some cases also designed for capturing interspecific variability (Adams et al., 2022; Klymus et al., 2020; Zanovello et al., 2023), and multi-species primers for metabarcoding studies (e.g., Hu et al., 2022; Zhang et al., 2020). However, as argued by Vences et al. (2012), single markers might have a significant failure rate (5–50%) when targeting wide taxonomic ranges. Using the mitochondrial gene Cytochrome B, we designed seven new primer pairs, and their performance and specificity were assessed (a) in silico against an amphibian reference database that included all species from the target genera; (b) in vitro using DNA extracts from tissue samples of most target species; and (c) in situ using water samples collected at 38 freshwater sites in the Province of Trento, Italy.

The second part of this study was motivated by the need to better understand the efficiency of various pipelines for processing eDNA metabarcoding data, in particular when intraspecific diversity is analyzed. Several tools are available (e.g., Elbrecht et al., 2018; Mousavi-Derazmahalleh et al., 2021; Wahlberg, 2019; Yoshitake et al., 2021), and a few comparisons among methods do exist (Flück et al., 2022; Straub et al., 2020), but little is known about the impact of parameter choice (Antich et al., 2021; Scott et al., 2018). Here, we aimed to provide additional evidence useful for selecting the appropriate bioinformatic approach in further studies.

2 | MATERIALS AND METHODS

2.1 | Study species

Our study focused on 12 amphibian species with resident breeding populations in the Province of Trento (Italy), which include seven Anurans and five Urodeles (Caldonazzi et al., 2002).

Anurans include *Rana temporaria* (Linneaus, 1758), *Rana dalmatina* (Fitzinger, 1839), *Bombina variegata* (Linneaus, 1758), *Bufo bufo* (Linnaeus, 1758), *Bufotes viridis* (Laurenti, 1768), and the pool frog, a species complex (*Pelophylax* kl. *esculentus* Linnaeus, 1758) that includes *Pelophylax lessonae* (Camerano, 1882) and *Pelophylax ridibundus* (Pallas, 1771) and their hybridogenetic form *P.* kl. *esculentus*. Since *P. lessonae* and the hybridogenetic form are sympatric, almost indistinguishable morphologically (Bovero et al., 2014; Lanza et al., 2009), and traditionally referred to as a complex, in the present paper they are considered as such, following the classification proposed in Caldonazzi et al. (2002). Lastly, here we referred to the treefrog populations in the study area as *Hyla intermedia* (Boulenger, 1882), although some authors have proposed the status of species for this taxon (*H. perrini*; Dufresnes et al., 2018).

The Urodeles found in the Province of Trento include *Salamandra salamandra* (Linnaeus, 1758), *Salamandra atra* (Laurenti, 1768), and *Ichthyosaura alpestris* (Laurenti, 1768). In addition, *Lissotriton vulgaris* (Linnaeus, 1758) occupies the southern part of the Province, while *Triturus carnifex* (Laurenti, 1768) is localized in the area surrounding the 'Laghetti di Marco' (Caldonazzi et al., 2002).

2.2 | Primer design and adequacy tests

Cytochrome B (hereafter, CytB) was chosen as our 'barcode' region as it presents a good trade-off between identifying conserved regions for primer binding, and the potential to provide information on both species and population genetic diversity, as well as phylogeographic patterns. CytB is also one of the most frequently used

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genes for amphibian metabarcoding studies, especially those that target both Anuran and Urodeles species (e.g., Cannon et al., 2016; Goldberg et al., 2011; Thomsen et al., 2012). Other commonly used markers such as 12S (Valentini et al., 2016) could have been adequate for species identification as well, but lacked the variability needed for an appropriate intraspecific diversity assessment. Moreover, considering the five major vertebrate taxa, CytB has more records in GenBank compared to other widely used markers such as Cytochrome Oxidase subunit I or 12S (van den Burg et al., 2020). Lastly, an online, open-access database is available for CytB curation, with a deployed script for updates (van den Burg et al., 2020).

Seven primer pairs were designed using Primer3Plus (Untergasser et al., 2012) with sequences downloaded from the ACDC Database (van den Burg et al., 2020), except for the forward primer of the pair 'Bufo' which is a modification of the primer Cyt Bufo F developed by Recuero et al. (2012). The resulting primer pairs amplify fragments of variable length, the shortest being 277 base pairs (hereafter, bp) long, and the longest 379 bp; they also target different amphibian families, subfamilies or genera, as reported in Table 1. The relative position of these markers with respect to the CytB gene of one of the target species (*R. temporaria*) is shown in Figure S1.

The primer pairs were tested in silico with ecoPCR (Ficetola et al., 2010). First, we used the RScript published by van den Burg et al. (2020) to create a database of CytB sequences for all genera of the target species (namely, *Bombina*, *Bufo*, *Bufotes*, *Hyla*, *Ichthyosaura*, *Lissotriton*, *Pelophylax*, *Rana*, *Salamandra* and *Triturus*) available in NCBI (download at 2023/01/18). The FASTA sequences were converted in an ecoPCR database format, and the two available CytB sequences of *H. perrini* (Dufresnes et al., 2018), which currently does not have a NCBI taxid entry, were added with the command *obitaxonomy* using the most recent common ancestor taxid (*Hyla*, thus 8421). An in silico PCR was run for each primer pair using default parameters (i.e. allowed number of mismatches equal to zero), and their taxonomic specificity and resolution were measured with a custom script in R.

The primers were then tested in vitro on DNA extracted from tissue samples of the target species available at the Fondazione E. Mach from previous studies (*B. variegata*, *B. viridis*, *I. alpestris*, *R. temporaria*, *R. dalmatina*, and *S. atra*) or collected from museum specimens (*H. intermedia* from the Civic Natural History Museum of Morbegno, and *P. kl. esculentus* from MUSE – Science Museum of Trento) preserved in 70% ethanol, and amplification success was confirmed by screening on a Qiaxcel Advanced System (Qiagen, Germany). For four of the species living in the study area, namely *B. bufo*, *L. vulgaris*, *S. salamandra* and *T. carnifex*, tissue samples were not available, and the test was not possible. Nonetheless, all the primer pairs were tested on at least one target species, as well as on the other species available. The final amplification mixture and PCR program for each primer pair are reported in Table S1. The amplification products were purified following the ExoSAP protocol (Thermo Fisher Scientific, USA) sequenced on an ABI 3130xl Sequencer (Thermo Fisher Scientific, USA), analyzed with Sequencher 5.4.6 (DNA sequence analysis software, Gene Codes

TABLE 1 Summary of primer pair characteristics.

Marker name	Frag. Length (bp)	Family/subfamily/genus	Target species	Reference	Forward primer	Reverse primer
Bomb	379	Bombinatoridae	*B. variegata*	This study	AAYGGAGCCTCYTTCTTCTT	AAGTABGGGTGAAATGGRAT
Bufo	356	Bufonidae	*B. bufo*, *B. viridis*	Recuero et al. (2012), modified	ATCTAYCYWCACATTGGACGAG	TTATAGGARTAGTARGCRTGGAA
Hyla	332	Hylidae	*H. intermedia*	This study	AACGGRGCCTCATTYTTYT	GGRTTRGAHGAGCCDGTTTG
Pelop	277	*Pelophylax*	*P. kl. esculentus*	This study	ATCGCMACMGGYYTATWY	AGGTTRGTRATTACWGTRG
Rana	305	*Rana*	*R. temporaria*, *R. dalmatina*	This study	ACTAAYCTYCTCTCMGCCG	GRTTRGCTGGYGTRAARTT
Sala	310	Salamandrinae	*S. salamandra*, *S. atra*	This study	CCVTCAAAYATYTCHTAYTG	AADGAYATTTGTCCTCAWGG
Trit	325	Pleurodelinae	*I. alpestris*, *L. vulgaris*, *T. carnifex*	This study	TGAGGRCAAATRTCVTTYTG	AAGTTTTCTGGRTCYCCRAG

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Corporation, Ann Arbor, MI USA) and assigned to the corresponding taxonomy with BLAST (Altschul et al., 1990) to ensure the correct amplification of the target sequence.

For in situ testing, water samples were collected during the peak reproductive period for amphibians (March–July, 2021) from 38 wetland sites in the Autonomous Province of Trento (Italy) in the eastern Alps. At each site, two to four spatial replicates were collected, depending on the wetland surface area, to capture as much taxonomic and genetic diversity as possible. Each replicate was sampled manually from just under the water surface using a sterile plastic canister, to avoid stirring up the sediment. The sampling protocol followed Zanovello et al. (2023). Briefly, the water collected in the canister was drawn up with a 100 mL syringe and filtered through two Sterivex GP Filter units (pore size 0.22 μm, Millipore cat. no. SVGPL10RC) until the filters clogged (40 mL to 800 mL). All filters were kept at ambient temperature for transport to the Fondazione E. Mach (FEM) the same day, and stored at −20°C until DNA extraction. All laboratory procedures were performed under BSL2 biological hoods at the Animal, Environmental and Antique DNA Platform at FEM, and followed recommended guidelines for eDNA analyses (Goldberg et al., 2015; Harper et al., 2019). DNA extraction was performed using the DNeasy PowerWater Sterivex Kit (Qiagen, Germany), following the manufacturer's instructions with minor modifications, as reported in Zanovello et al. (2023). The two filters corresponding to the same spatial replicate were processed simultaneously and their extracts were merged into a single tube at the final step of the protocol. DNA extraction involved batches of a maximum of 12 water replicate samples, including one negative control (extraction blank) for each batch. All extracts and extraction blanks were PCR amplified with each primer pair according to the respective amplification mixture and PCR program (see Table S1). The amplification success of the samples was confirmed via screening on a Qiaxcel Advanced System (Qiagen). One PCR was run per replicate, and a second PCR was run only if the first one showed no amplification bands. One negative control (PCR blank) was also included for each PCR reaction. All successful amplification products along with 8 negative controls that showed signs of contamination on the Qiaxcel, and 12 more, randomly chosen, negative controls were then purified with the MinElute PCR Purification Kit (Qiagen) following manufacturer's instructions. Each purified product was sequenced at the FEM Sequencing and Genotyping Platform using paired-end sequencing (2 × 300 bp) on an Illumina Miseq (Illumina, San Diego, CA) with a 30,000 bp coverage.

2.3 | Bioinformatic workflows design and application

Two bioinformatic tools were applied for processing raw sequence files, MICCA (Albanese et al., 2015) and OBITools (Boyer et al., 2016), and three tools for taxonomic assignment, VSEARCH (Rognes et al., 2016), ecotag (Boyer et al., 2016) or metabinkit (Fonseca & Egeter, 2023), resulting in three different bioinformatic pipelines (MICCA+VSEARCH, OBITools+ecotag and OBITools+metabinkit, hereafter, MV, OE and OM pipelines). A summary of pipeline steps and parameters can be found in Figure 1. Both MICCA and OBITools have previously been used in eDNA metabarcoding studies (e.g., Li et al., 2021; Lopes et al., 2017; Valentini et al., 2016; Zanovello et al., 2023). The VSEARCH-based consensus classifier implemented in MICCA searches the database for each query sequence and retrieves up to a user-defined number of hits above the given identity threshold. Then, VSEARCH assigns to each query the most specific taxonomic label that is associated with at least a user-defined number of the hits. In the case of metabinkit, first the query sequences are aligned to the reference database. For each query the alignments are then filtered based on the defined percentage identity thresholds, and the lowest common ancestor is determined for all alignments passing the filters, thus determining the assigned taxon. Ecotag first searches the database for the reference sequence(s) with the highest similarity to the query, then retrieves all other reference sequences whose similarity to the first reference sequence(s) is equal to or greater than the similarity between the first reference and the query. Finally, it assigns the query sequence to the first taxa found in common between the first and second reference sequences, going back to higher taxonomic levels if needed. More details on VSEARCH, ecotag, and metabinkit classifier algorithms can be found in their respective manuals (Boyer et al., 2016; Fonseca & Egeter, 2023; Rognes et al., 2016).

At the merging step of the pipeline MV, sequences were aligned only if overlapping for at least 100 bp and with no more than 10 mismatches within the overlapping region. In the OBITools pipelines (OE and OM) sequences were not merged if the alignment score was below 40. After the primer trimming step, MICCA gives the option to filter the sequences according to a maximum allowed expected error (EE) rate % and minimum length based on the expected target fragment characteristics, while OBITools allows both minimum and maximum expected length for filtering to be set. All pipelines had a denoising step performed with UNOISE3 (Edgar, 2016), an algorithm that preserves intra-cluster variability (Antich et al., 2021) and included the removal of chimeric fragments based on the default abundance skew (16). The sequences were not clustered to avoid losing information on exact sequence variants (hereafter, ESVs), and thereby potentially true haplotypes (Antich et al., 2021; Callahan et al., 2017; Porter & Hajibabaei, 2020). For taxonomic identification, sequences resulting from MICCA were assigned with VSEARCH using a similarity percentage threshold of 95%. Those from OBITools were classified with ecotag also using the default threshold of 95%, whereas with the package metabinkit a 97% blast identity was used as a threshold for species determination and 90% for genus and family, allowing a range of 2% above and below the threshold to build a consensus. The reference database used for taxonomic assignments corresponded to that used for the in silico testing of marker performance. The final step, removal of contaminations based on negative controls and rare taxa, was common to all pipelines and was performed using a custom R script (R Core Team, 2023; see Figure 1 for parameters used). The number of reads and/or ESVs retained after

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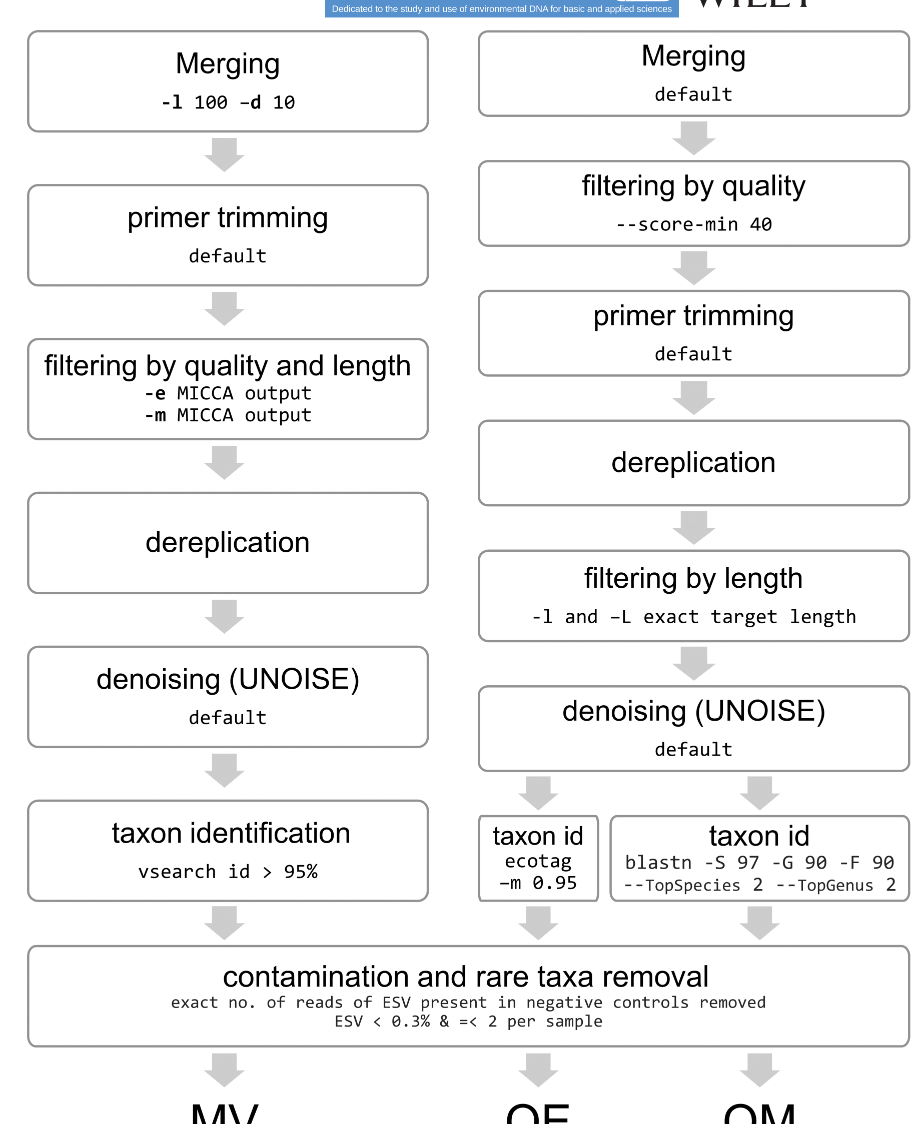

FIGURE 1 Summary of the bioinformatic steps and parameters for each pipeline: MV, MICCA+VSEARCH; OE, OBITools+ecotag; OM, OBITools+metabinkit.

each bioinformatic pipeline processing is shown in Table S2. For each pipeline and each marker, we calculated the proportion of reads and ESVs assigned to the target amphibian species, non-target amphibian species, and unassigned ESVs.

3 | RESULTS AND DISCUSSION

3.1 | In silico, in vitro and in situ primer evaluation

Despite the presence of degenerated nucleotides, the in silico PCR showed that the majority of the primer pairs designed here amplify, among amphibians of the considered genera, only species from the target taxa, except for the markers Pelop, Sala and Trit. In fact, Pelop appears to bind to several species of the Ranidae family, thus it could in principle be applied in monitoring studies targeting the whole family. The last two markers, Sala and Trit, are both likely to work well for species of the genus *Salamandra* and also amplify some Pleurodelinae species. Since the reference database did not include all European species of amphibians, it is also possible that the primers are useful for detecting additional species.

Regarding their taxonomic resolution, for five out of 12 amphibian species living in the Province of Trento, the respective marker allowed the correct taxonomic assignment of all available sequences, as shown in Table 2. The sequences of an additional species, *L. vulgaris*, were consistently identified by the marker Sala (and not by Trit). The sequences of *R. temporaria* were correctly identified by both Pelop and Rana markers, as occurred for the two *Salamandra* species with marker Trit. The sequences of *B. variegata* were correctly classified in 97%, those of *B. viridis* in 94% and *H. intermedia* in 96% of cases. In these cases, we argue that misassignment of the original sequences available in the GenBank database could explain the presence of incorrectly identified sequences (e.g., Bagheri et al., 2020; Schnoes et al., 2009). The genus *Pelophylax* had the lowest score in terms of resolution (*P. lessonae* 67%, *P. ridibundus* 50%). These species might represent a special case, as most of these (and their hybrids) are known to be almost indistinguishable morphologically (Di Nicola et al., 2019), thus possibly leading to taxonomic

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TABLE 2 Results of primer tests in silico, in vitro and in situ: x: cases in which the marker was positive for the species; *: percentage in silico test below 100%; n/a test not possible.

	Species & markers	*B. variegata*	*B. bufo*	*B. viridis*	*H. intermedia*	*I. alpestris*	*L. vulgaris*	*P. kl. esculentus*	*R. dalmatina*	*R. temporaria*	*S. atra*	*S. salamandra*	*T. carnifex*
In silico	Bomb	x*											
	Bufo		n/a	x*									
	Hyla				x*								
	Pelop							x*		x			
	Rana								x	x			
	Sala						x				x	x	
	Trit					n/a					x	x	x
In vitro	Bomb	x	n/a				n/a				x	n/a	n/a
	Bufo		n/a	x			n/a					n/a	n/a
	Hyla		n/a	x	x		n/a	x	x	x		n/a	n/a
	Pelop		n/a	x	x		n/a	x	x	x		n/a	n/a
	Rana		n/a	x	x		n/a	x	x	x	x	n/a	n/a
	Sala		n/a			x	n/a			x	x	n/a	n/a
	Trit	x	n/a			x	n/a	x	x	x	x	n/a	n/a
In situ	Bomb	x								x 2,3			
	Bufo		x										
	Hyla		x 3							x 3			
	Pelop							x	x	x			x 3
	Rana								x	x			
	Sala					x	x 1, 2			x 2, 3	x	x	x
	Trit					x				x		x	

Note: For the in situ test, numbers correspond to the pipeline that detected the species: 1: MV, 2: OE, 3: OM, otherwise all pipelines detected the species for the specific marker.

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assignment issues concerning the original specimens that were used for evaluation of the primers. Moreover, it should be noted that for some *Pelophylax* taxa the status of species or subspecies is still debated (e.g., *P. kurtmuelleri*, see Di Nicola et al., 2019). Lastly, for two species (*B. bufo* and *I. alpestris*) the test did not give any results, as the available sequences did not contain the primer binding sites. The results for other species included in the in silico test but not present in the study area can be found in Table S3.

For in vitro tests, the primer pairs showed 100% amplification success for all the respective target species for which tissue samples were available (as shown in Table 2). Apart from the marker Bufo, the primers also amplified one (in the case of marker Bomb) or more non-target amphibian species. In fact, some degenerated bases were included in the primers as we aimed to target as many species as possible with the same marker. In addition, the PCR mix and program conditions were very permissive (e.g. allowing up to 55 cycles of PCR reaction, see Table S1), in order to overcome potential barriers to amplification success, such as the presence of PCR inhibitors, as well as the occurrence of degraded DNA fragments, in both museum and field samples. Most of the non-target amphibian sequences we obtained were of overall lower quality (data not shown), plausibly as a result of the PCR conditions used.

Regarding the in situ application of the protocol, all markers but one were able to detect at least one of the target species. The only exception was the Hyla marker, which did not detect species of Hylidae in any of the water samples. Unfortunately, no recent traditional survey data was available for species of this Family in our study area for confirmation of this result. However, according to research-level reports from iNaturalist (iNaturalist.org, 2023), *H. intermedia* was confirmed as present in the Province during our sampling period from only three casual observations, one of which referred to one of our sites. Considering the rarity of this species, it is possible that our protocol is not sensitive enough to detect it, an issue already highlighted by other eDNA-based monitoring studies (e.g., Pope et al., 2020). *B. viridis* was also expected in the study area but was not detected by our protocol. In this case, the false negative can be explained by both its relatively limited distribution and the peculiar habitat preferences of the species, that has adapted to living in anthropized areas (Caldonazzi et al., 2002). In fact, the vast majority of iNaturalist reports for *B. viridis* in the Province were from urbanized areas that were not included in our sampling design.

Apart from Bufo and Rana markers, all markers detected non-target amphibian species at least in one of the pipelines used (see Table 2), so combinations of fewer markers could detect all desired species, making the protocol economically efficient. For instance, the primer pair Pelop captured all the species of the family Ranidae known for the study area, detecting one ESV for *R. dalmatina* (compared to one detected with Rana) and eight (MV) to 11 (OE and OM) ESVs of *R. temporaria* (compared to three for MV and 11 for OE and OM, with marker Rana). Therefore we suggest using Pelop for detection of both genera, *Rana* and *Pelophylax*. Similarly, Sala outperformed Trit, which was designed on sequences of Italian Pleurodelinae species, in terms of number of Pleurodelinae taxa detected, identifying all the newts present in the Autonomous Province of Trento. Therefore, since the target regions of markers Sala and Trit overlap almost entirely, for future applications where the target species belong to the family Salamandridae, we recommend using the first primer pair. Lastly, the difference in the length of our markers did not seem to determine differences in the number of target and non-target ESVs detected in our dataset, although this occurrence has been documented in other studies (Andres et al., 2023, and references therein).

3.2 | Impact of bioinformatic pipelines on detecting target and non-target ESVs from eDNA samples

For each pipeline and each marker, the absolute numbers and proportion of reads and ESVs assigned to the target amphibian species, non-target amphibian species and unclassified taxa are shown in Table 3. Overall, with the exception of marker Bomb, the MV pipeline retained the highest number of reads (Table S2) in all three taxonomic categories. However, the proportion of reads assigned to target amphibians by MV was lower than that of the other two pipelines even if the absolute number was still higher. Regarding the number of identified ESVs, the three pipelines showed similar results for Bomb, Bufo and Sala markers. MV was able to retrieve more target haplotypes for the Pelop marker, while the two OBITools pipelines (OE and OM) outperformed MV in the case of Rana and Trit markers. None of the pipelines identified haplotypes of the target species *H. intermedia* with the Hyla marker, although OM detected five ESVs belonging to other amphibian species. The proportion of ESVs assigned to the target species by MV over the total number of ESV was lower than the other two pipelines, while OE and OM assigned a similar proportion of reads and ESVs to target or non-target amphibian species. In particular, the OM pipeline retained the most non-target amphibian ESVs. This variation in the detection of non-target sequences across pipelines was expected due to the different taxonomic assignment methods used. Notably, the differences in number of reads and ESVs between pipelines is not consistent for all markers. While we applied the default parameters whenever possible, it should be noted that in the MV pipeline the expected error rates allowed at the filtering by quality step varied between markers, corresponding to 0.25% for all markers except Bufo and Sala (0.5%), thus possibly producing differences between markers in the MV dataset.

Concerning the difference in terms of ESVs, it should be noted that the extra ESVs retrieved with OBITools could be errors, and not true haplotypes. In order to address this issue, we applied throughout our workflow several of the best practices for identification and removal of erroneous sequences summarized by Andres et al. (2023), such as a sampling design focusing on locations and times of the year where the target species are known to be present, application of a denoising and chimera removal step during raw reads processing, and of a sequence similarity threshold to known (reference) sequences for taxonomic assignment, and lastly removal of low frequencies

TABLE 3 Number of reads and ESVs assigned by the three pipelines to the target amphibian species, non-target amphibian species, and unclassified taxa for each marker.

	Bioinformatic pipelines											
	MV				OE				OM			
Marker name	N. Reads	% reads	N. ESVs	% ESVs	N. Reads	% reads	N. ESVs	% ESVs	N. Reads	% reads	N. ESVs	% ESVs
Bomb												
Target Amphibia	3636	39.53	2	6.06	8100	80.65	2	40	8100	80.65	2	40
Unclassified	5562	60.47	31	93.94	1944	19.35	3	60	1944	19.35	3	60
Bufo												
Target Amphibia	106,842	95.56	4	23.53	7837	99.10	3	60	7837	99.10	3	60
Unclassified	4962	4.44	13	76.47	71	0.90	2	40	71	0.90	2	40
Hyla												
Non-target Amphibia									7479	55.38	5	31.25
Unclassified	50,688	100	106	100	13,505	100	16	100	6026	44.62	11	68.75
Pelop												
Target Amphibia	14,374	16.57	5	11.36	4675	12.54	1	4.54	4675	12.54	1	4.54
Non-target Amphibia	57,162	65.91	9	20.46	23,759	63.75	12	54.54	32,270	86.60	18	81.82
Unclassified	15,199	17.52	30	68.18	8832	23.70	9	40.91	321	0.86	3	13.63
Rana												
Target Amphibia	38,640	60.24	4	1.42	23,475	79.58	12	41.38	23,475	79.59	12	41.38
Non-target Amphibia									12	0.04	1	3.45
Unclassified	25,500	39.76	277	98.58	6022	20.42	17	58.62	6010	20.38	16	55.17
Sala												
Target Amphibia	5733	4.12	3	1.29	1976	4.13	3	3.61	1976	4.13	3	3.61
Non-target Amphibia	47,571	34.19	16	6.87	17,533	36.64	23	27.71	21,657	45.25	31	37.35
Unclassified	85,850	61.69	214	91.84	28,347	59.23	57	68.68	24,233	50.62	49	59.04
Trit												
Target Amphibia	28,287	22.35	5	4.42	2832	14.65	13	19.70	2832	14.65	13	19.70
Non-target Amphibia	57,177	45.18	8	7.08	7800	40.35	13	19.70	7800	40.35	13	19.70
Unclassified	41,072	32.46	100	88.50	8699	45	40	60.60	8699	45	40	60.60

Abbreviations: MV, MICCA+VSEARCH; OE, OBITools+ecotag; OM, OBITools+metabinkit.

ESVs. Despite these precautions, undetected chimeric sequences (Edgar, 2016) and/or sequencing errors (Nakamura et al., 2011; Schirmer et al., 2015) could still be present in the dataset, leading to false positive errors (Andres et al., 2023). For most of our target species, up-to date field genetic data for the selected gene marker are scarce or not available (for the most recent studies concerning the alpine region see Cornetti et al., 2016; Canestrelli & Nascetti, 2008; Veith et al., 2003; Pichlmüller et al., 2013; Riberon et al., 2002; Canestrelli et al., 2006). Nevertheless, overall, our markers showed patterns of genetic diversity compatible with tissue-based genetic studies. For instance, Cornetti et al. (2016) found that *B. variegata* populations have low mitochondrial diversity in the study area, reporting only four haplotypes from nine sampling sites (from 200 individuals in total). As marker Bomb identified two ESVs in water samples from two sites, our results appear in agreement with this previous study. Another traditional study based on Cytochrome Oxidase I (COI) diversity found 12 *R. temporaria* haplotypes in the Province of Trento, only one of which (CA2) was distributed across the Province, while the others showed a clear separation due to the presence of the Adige river (Marchesini et al., 2017). Here, the marker Pelop showed more consistent results across pipelines, as it detected eight *R. temporaria* haplotypes according to pipeline MV, and 11 for both OE and OM, while Rana retrieved only three haplotypes with pipeline MV, but again 11 with OE and OM. The combination of Pelop and MV, in particular, yielded the most similar diversity pattern with respect to the traditional study, with only two CytB haplotypes found

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in sites located on the opposite sides of the Adige Valley, and the other six that were found in only one side. On the other hand, the 11 haplotypes of OE and OM were each detected in one site only, and therefore no genetic structure emerges from these data. In an effort to further validate our ESVs, the sequences were also compared with all available haplotypes in Genbank. According to this comparison, 16 out of the 56 amphibian (target and non-target) ESVs retrieved by MV were identical to already known haplotypes, as well as 14 out of 79 for OE and 17 out of 88 for OM. Overall, MV could be the most suitable pipeline if the aim is to retrieve the highest number of reads possible, although this comes at the cost of being more computationally demanding as it is less efficient in removing non-target reads. In addition, MV also appears to be the safest choice for decreasing the probability of false positive errors in the dataset, as it had the best rate of Genbank-confirmed over total ESVs, even though the pipeline could also be losing some true genetic information, given its more conservative algorithm for retrieving ESVs.

4 | CONCLUSIONS

The new eDNA workflow presented here proved successful in detecting almost all the target species that were expected in our study area, while at the same time providing information on their mitochondrial genetic diversity. This new marker set combined with MV has the potential to become a standard monitoring tool for the Alpine region and beyond, with the capacity to collect both inter- and intra-specific diversity data in a completely non-invasive framework.

However, the debate about which pipeline would be more efficient for characterizing population genetic diversity is still open. The problem of false positives and negatives in eDNA-based multi-species surveys is known and discussed (e.g., Cristescu & Hebert, 2018), and it is an issue that requires even more attention in intra-specific diversity studies (Adams et al., 2022; Elbrecht et al., 2018; Macé et al., 2022). Additional studies comparing bioinformatic pipelines in their ability to retrieve in eDNA data the correct number and sequence of haplotypes, based possibly also on traditional sampling as controls, are urgently required.

AUTHOR CONTRIBUTIONS

Conceived and designed the study: LZ, GM, MFMS, PB, PP, GB, HCH; acquired the funding: GM, PP, GB, HCH; collected the samples: LZ, GM, SC, GG; performed laboratory analyses: LZ, GM; completed bioinformatic analyses: LZ, MFMS; drafted the manuscript: LZ, MFMS, HCH; read, commented and approved the manuscript: all authors.

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CONFLICT OF INTEREST STATEMENT

The authors declare no competing interests.

DATA AVAILABILITY STATEMENT

Sequences available upon request. Correspondence and requests for materials should be addressed to H.C.H. (email: heidi.hauffe@fmach.it).

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