Alpine freshwater fish biodiversity assessment: an inter-calibration test for metabarcoding method set up

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ABSTRACT

The analysis of environmental DNA (eDNA) by high throughput sequencing (HTS) is proving to be a promising tool for freshwater fish biodiversity assessment in Europe within the Water Framework Directive (WFD, 2000/60/EC), especially for large rivers and lakes where current fish monitoring techniques have known shortcomings. These new biomonitoring methods based on eDNA show several advantages compared to classical morphological methods. The sampling procedures are easier and cheaper and eDNA metabarcoding is non-invasive and very sensitive, allowing for the detection of traces of DNA. However, eDNA metabarcoding methods need careful standardization to make the results of different surveys comparable. The aim of the EU project Eco-AlpsWater is to test and validate molecular biodiversity monitoring tools for aquatic ecosystems (*i.e.*, eDNA metabarcoding) to improve the traditional WFD monitoring approaches in Alpine waterbodies. To this end, an inter-calibration test was performed using fish mock community samples containing either tissue-extracted DNA, eDNA collected from aquaculture tanks and eDNA samples collected from Lake Bourget (France). Samples were analysed using a DNA metabarcoding approach, relying on the amplification and HTS of a 12S rDNA marker, in two separate laboratories, to evaluate if different laboratory and bioinformatic protocols can provide a reliable and comparable description of the fish communities in both mock and natural samples. Our results highlight good replicability of the molecular laboratory protocols for HTS and good amplification success of selected primers, providing essential information concerning the taxonomic resolution of the 12S mitochondrial marker in describing the Alpine fish communities. Interestingly, different concentrations of species DNA in the mock samples were well represented by the relative DNA reads abundance. These tests confirm the reproducibility of eDNA metabarcoding analyses for the biomonitoring of freshwater fish inhabiting Alpine and peri-Alpine lakes and rivers.

INTRODUCTION

Since its first application, the use of environmental DNA (eDNA) isolated from water samples to detect the presence of taxa has been considered a promising method to improve aquatic biomonitoring (Ficetola *et al.*, 2008; Goldberg *et al.*, 2015; Lawson Handley, 2015). Environmental DNA is the genetic material present in different environmental matrices such as sediment, water, and air, and belonging to the organisms inhabiting the surveyed area; it includes DNA released in the environment (intra or extracellular) and DNA taken directly from living cells (Pawlowski *et al.*, 2018). This eDNA can be extracted from the environmental matrices and used to assess community biodiversity through the amplification of a short DNA region used as a "barcode" (Hebert *et al.*, 2003; Taberlet *et al.*, 2012; Ward *et al.*, 2009) and sequenced with high-throughput sequencing techniques (*i.e.*, metabarcoding, Kuntke *et al.*, 2020; Schenekar *et al.*, 2020). The use of eDNA metabarcoding can significantly improve biodiversity monitoring surveys through the early detection of exotic and potentially invasive species and the tracking of elusive endangered species (Deiner *et al.*, 2018; Pawlowski *et al.*, 2018; Taberlet *et al.*, 2018).

A range of organisms is used worldwide as indicators (Biological Quality Elements (BQEs)) to monitor the quality status of aquatic ecosystems, namely phytoplankton, phytobenthos, aquatic plants, macroinvertebrates, and fish (European Environment Agency, 2018). The sampling method officially recommended for the biodiversity assessment of river and lake fish by the Water Framework Directive (EN14011, 2003) are electrofishing and gill netting, which are quite expensive methods and require a large and qualified staff to be performed. Moreover, several sampling practices (*e.g.*, seines or trawling) can severely damage the habitat and in large lakes can heavily affect fish abundance (Irvine *et al.*, 2019; Njiru *et al.*, 2018).

Since 2000, the European Union has been actively engaged in the protection and enhancement of aquatic ecosystems: freshwater biomonitoring promoted by the implementation of the EU Water Framework Directive (WFD) (European Commission, 2000) allows for the evaluation and improvement of their quality status. However, classical biomonitoring practices require good taxonomic expertise and the probability of detecting species that represent <1% of the total abundance is

very low (Paller, 1995), thus providing partial estimates for entire communities (Deiner *et al*., 2017). Compared to electrofishing and gill netting procedures, water sampling procedures for eDNA analyses proved to be potentially cheaper and easier, non-invasive, and suited for surveys in extremely difficult sites. Reduced operational costs could allow for regular sampling during the year, providing time-series data and a systematic monitoring of fish biodiversity (and, in general, of community diversity) in different seasons and extreme events (extremely dry seasons or floods). Conversely, traditional sampling can usually be performed once or twice a year.

Standardization of all the protocols, from the sampling activities to the taxonomic assignment of DNA sequences (Dickie *et al*., 2018), is paramount to allow for the comparability among eDNA metabarcoding studies for the ecological assessment of habitats or ecosystems (Goldberg *et al*., 2015) and to improve the sensitivity of metabarcoding assays. The use of experimental controls and mock communities allows to exclude unspecific signals and verify the recovery of species signals and quantitative representation of species in the samples, as well as to determine bioinformatic filtering steps, and threshold levels. Using standardized methods allows for a better interpretation of ecosystem response to pressures (Mock and Kirkham, 2012; Morales and Holben, 2011) however, each study has its specificity and often requires a customization of the metabarcoding protocols that often need further validation, especially when universal polymerase chain reaction (PCR) primers are used to explore fish community diversity (Bylemans *et al*., 2018; Thalinger *et al*., 2021a).

Pilot studies, comprising inter-calibration tests, are an invaluable tool to evaluate if different approaches can lead to similar and comparable results (Zinger *et al*., 2019). It is for these reasons that, in this study, we performed an inter-calibration exercise involving two different laboratories to evaluate the reproducibility and possible limits of an eDNA metabarcoding procedure to describe the fish community diversity in Alpine lakes and rivers. No previous studies were performed to estimate Alpine fish biodiversity by using eDNA metabarcoding, and a pilot test was highly recommended. Mock samples made of tissue DNA pools as well as aquaria water samples and lake water samples were included in the test to compare the species detection performance and replicability of the method. This test has been conceived within the Eco-AlpsWater network, a project funded by the European Union, whose ultimate goal is to evaluate and validate emerging technologies based on eDNA metabarcoding for the biodiversity assessment of freshwater ecosystems in the Alpine region (https://www.alpine-space.eu/projects/eco-alpswater).

MATERIALS

Set-up of mock and environmental samples

All the samples were prepared at INRAE (Institut National de Recherche pour l'Agriculture, l'Alimentation et l'Environnement) as a blind test for the laboratories involved in the inter-calibration exercise. A negative control sample (pure water filtered following the same protocol used for the other samples) was included in the test and analysed following the same procedures (DNA extraction, library preparation, and sequencing) used for the other samples.

Three mock samples including an increasing number of species pooled (6, 9, and 14, hereafter M6, M9, and M14) and different DNA proportions (Table S1) were set up using genomic DNA extracts from fin clips. We used one individual per fish species to set up these three mock samples. The fish species used to assemble these mock samples are species commonly found in lakes and rivers of the Alpine and perialpine regions. A fourth mock sample was prepared by collecting 100 mL of water from each of ten fish tanks containing one single species each (for a total of seven species, namely *Salmo carpio* L., *Oncorhynchus mykiss* W., *Barbus caninus* B., *Perca fluviatilis* L., *Lepomis gibbosus* L., *Carassius carassius* L., and *Tinca tinca* L.). For each tank, we collected the same volume of water to obtain a total volume of 1 L to simulate a real environmental sampling procedure. This sample was collected on the 3rd of December, 2019, and extracted on the 9th of December, 2019. Moreover, a third set of three environmental samples of 6 x 2 L each were collected from three different areas in Lake Bourget (all subsurface samples, *i.e*., 10-20 cm; Figure 1). Three sampling points were sampled from each lake bank to simulate a transect and 2 L of water were collected from each site (Figure 1). After careful mixing of the samples, 1 L of water collected from the fish tank sample and Lake Bourget samples were filtered by using 0.45 µm Sterivex™ capsule filters (Merck Millipore, Burlington, USA); the filtration cartridges were filled with SPYGEN (SPYGEN, Le Bourget du Lac Cedex, France) preservation buffer and stored at room temperature until the DNA extraction step. These lake samples were collected on the 16th of October, 2019, stored at room temperature, and extracted on the 21st and 22nd of October, 2019.

DNA extraction and library preparation

For fin clips DNA extractions, we used the NucleoSpin® DNA RapidLyse kit from MACHEREY-NAGEL. eDNA extraction was performed using the NucleoSpin® Soil kit (MACHEREY-NAGEL, Allentown, USA) following the protocol described in Pont *et al*. (2018) and adapted to Sterivex™ capsule filters. Twenty µL of genomic DNA

extract were delivered to both Fondazione Edmund Mach, Italy (LAB_A) and NatureMetrics, UK (LAB_B) sequencing platforms for further laboratory processing.

For both laboratories, a hypervariable region of 12S rRNA was amplified via a two-step PCR process (Supplementary Information).

The two laboratories performed all PCRs in the presence of both a negative and a positive control (*i.e.*, a mock community with a known composition of fish species). Amplification success at each step was determined by gel electrophoresis. All PCRs replicates per sample were pooled and purified using CleanNGS beads (CleanNA, Waddinxveen, Netherlands) by LAB_A and MagBind TotalPure NGS (Omega Biotek, Norcross, USA) magnetic beads with a ratio of 0.8:1 (beads: DNA) by LAB_B, to remove primer dimers.

The sequences, saved in FASTQ formatted files, were deposited to the European Nucleotide Archive (ENA) with study accession number PRJEB49223.

Bioinformatic analyses

Bioinformatic analyses were performed by Lab_A using the OBITools3 software (Boyer *et al*., 2016). A reference database was created simulating a PCR amplification in silico by using the ecoPCR program: the whole vertebrate EMBL database (June 2020) and the MiFish-U primers were used, allowing for a maximum of three mismatches with the published sequences, and blocking the last two nucleotides in the primer sequence. For FASTQ Miseq sequences analyses the alignpairedend script was used to perform a micro-assembly of paired-end reads and sequences with Illumina FASTQ quality scores <30 across the head, tail, or total length of the sequence were discarded. The ngsfilter script was used to assign the reads to each sample through barcode identification and, after a dereplication step, only sequences longer than 80 nucleotides and a count ≥10 were retained for further analyses. The obiclean script was used to detect the potential PCR errors, selecting only sequences with the 'head' status and abundance higher than 0.05%. The taxonomic assignment was performed by using the ecotag script and the reference database, considering a 97% of similarity. The taxonomic assignment was further inspected by using BLASTn (Zhang *et al*., 2000) algorithm optimized for very similar sequences (megablast) on the nucleotide collection (nr/nt) that includes all GenBank + EMBL + DDBJ + PDB sequences when uncertainties in the identification emerged. Rarefaction analysis was performed on Lake Bourget sequences to evaluate if the sequencing effort allowed to reach a plateau using the vegan package (Oksanen, 2016) and the rarecurve function in R environment (R Core Team, 2020).

To evaluate the intra-individual variability of the 12S rDNA copies, both *Salmo trutta* Miseq sequences assigned at the species level and those assigned only at the genus level and obtained from the mock tissue sample (DNA extracted from the fin clip of a single individual), were further evaluated by using MEGAX (Kumar *et al*., 2018). These sequences were aligned separately by using the Muscle software (Edgar, 2004) with default parameters, and the mean distance among sequences was computed by using the Kimura 2-parameter model (K2P, Kimura, 1980) and 1000 bootstrap.

At LAB_B samples were demultiplexed based on the combination of the i5 and i7 index tags. Paired-end reads for each sample were merged with USEARCH (Edgar, 2010), with a minimum overlap of 20% of the total read length. Forward and reverse primers were trimmed from the merged sequences using cutadapt (Martin, 2011), and retained if the trimmed length was between 140 and 200 bp. These sequences were quality filtered with USEARCH to retain only those with an expected error rate per base of 0.05 or below, and dereplicated by sample, retaining singletons. Unique reads from all samples were denoised in a single analysis with UNOISE (Edgar and Flyvbjerg, 2015), requiring retained ZOTU`s (zero-radius OTU's) to have a minimum abundance of 8 in at least one sample. A taxon-by-sample table was generated by mapping all dereplicated reads for each sample to the ZOTU representative sequences with USEARCH, at an identity threshold of 97%. ZOTU's were identified via BLASTn (Zhang *et al.*, 2000) searches of the representative sequences against the whole nt database and a local curated database of 12S fish sequences. Identifications were based

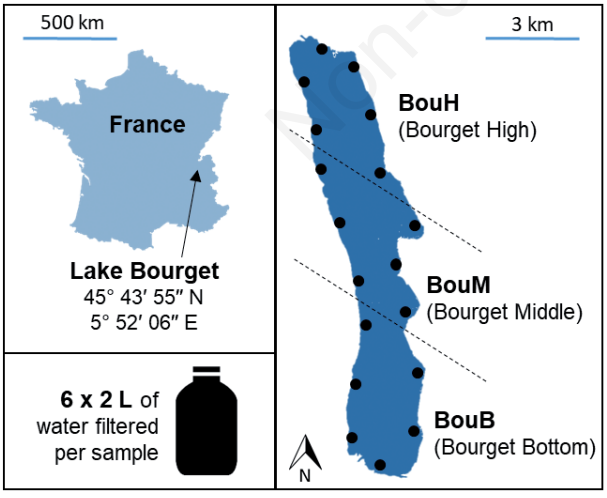

Figure 1. Sampling sites map of Lake Bourget. Three 6 x 2 L samples were collected in three different areas of the lake and 1 L of water was filtered to evaluate the effectiveness of the eDNA metabarcoding survey in describing the fish community diversity (BouB = low Lake Bourget, BouM = medium Lake Bourget, BouH = high Lake Bourget).

on the highest available percentage identity at 98–100%, with an e-score of $1e^{-20}$ and a hit length of at least 80% of the query sequence. In cases where multiple reference sequences matched equally to the query sequence then a more conservative higher taxonomic classification was considered. Only sequences with species- or genus-level identifications were included in the final results. When a species was represented by multiple ZOTUs, the one with the highest percentage match to that species was taken as the representative. Typically, the other sequences having the same occurrence pattern and the lower sequence similarity can be attributed to PCR or sequencing errors.

Data analysis

Analysis of covariance (ANCOVA) was used to compare two regression lines by testing the effect of a categorical factor (the two laboratories involved in the high throughput sequencing (HTS) analyses) on the dependent variable (fraction of taxonomic annotated sequences) while controlling for the effect of the continuous covariable (fraction of DNA of single species in mock tissue samples; Crawley, 2005). The results allowed to test differences in the regression slopes (interaction effect) and intercepts (main effects) in the two regression models (Kéry, 2010). Moreover, a regression analysis was performed by using the ggpubr function in R to compare the number of OTUs obtained by the two laboratories for the Lake Bourget and the data (fish number and biomass) collected from the traditional survey performed in Lake Bourget in 2018 (Table S11, Figure S2). Statistical analyses were computed using R 4.03 (R Core Team, 2020). For these analyses, only the sequences identified at the species or genus level by both Lab_A and Lab_B were considered.

RESULTS

LAB_A analyses produced 14,600,000 sequences and, after the quality control step, an average of 250,000 sequences per sample were obtained, excluding the negative control sample. After Obitools3 analyses, 1,500,000 sequences were assigned, and 20 species, 9 genera, and 3 families respectively were identified. The blank sample produced less than 5000 sequences that did not find any match with vertebrate sequences present in the EMBL database, with a similarity threshold of 97%.

LAB_B analyses produced an average of 320,000 sequences per sample (excluding the template negative control). After LAB_B quality control steps, an average of 270,000 sequences per sample were achieved. A total of 1,900,000 sequences after LAB_B bioinformatic analyses were successfully assigned, identifying 22 species of fish. The blank sample failed to amplify and yielded less than 500 sequences.

Tissue DNA mock samples

The identification success of the tissue DNA mock sample revealed good performance of both the LAB_A and LAB_B analyses, with few inconsistencies in the assignment of OTUs to *Coregonus*, *Esox*, *Cyprinus* and *Salmo* genera (Table 1). More in detail, *Coregonus lavaretus* L. sequences were assigned only at the genus level by Obitools3, because the 12S rDNA fragment is not diagnostic for species within this genus, and the ecotag script assigns a sequence to the most recent common ancestor when a sequence shows the same percentage of similarity with different species, as shown by a BLAST similarity search (Table S4): five different species were indeed identified with 100% of identity. Moreover, some of these sequences were assigned only at the family level, suggesting the presence of different genera among the reference sequences showing a level of similarity higher than 97% (as proved by a BLASTn similarity search, Table S5). The same sequence was assigned to 14 different species belonging to the *Coregonus* genus and one species belonging to the *Stenodus* genus, with a percentage of identity between 98 and 99%. The same inconsistencies are found for *Cyprinus*, *Esox*, *Salmo* and *Silurus* (Tabs S6, S7, S8 and S9).

Some *Squalius cephalus* sequences were assigned to *Leuciscus* sp. because of a limited resolution power of the 12S fragment, as revealed by a Blastn search, revealing a sequence similarity between 98 and 99% to both *Squalius cephalus* and *Leuciscus leuciscus* of sequences identified as *Leuciscus* sp. LAB_B assigned a few Coregonus sequences to *Coregonus maraena* B., which is a species distributed in the Baltic Sea basin and was not included in the mock sample (Table 1). Furthermore, a wrong *Salvelinus* species was identified (*Salvelinus fontinalis* M.) instead of the expected *Salvelinus alpinus* L. Notably, three species present in very low proportions (Table 1) in the M14 mock sample were not detected by both the LAB_A and LAB_B procedures, namely *Gobio gobio* L., *Esox lucius* L. and *Lota lota* L.

The comparison between the DNA proportions included in the mock samples for each species and the DNA sequences proportions retrieved using HTS for these species showed a good correlation (for both laboratories, Figure 2). This was also confirmed for the mock assemblage with the most complex species composition (M14). In the three mock assemblages, the individual slopes were always significant ($p<0.001$) and ranged between 0.8 and 1.1. Moreover, both slopes and intercepts did not show significant differences ($p>0.05$).

The mean genetic K2P distance computed among the *Salmo trutta* L. sequences assigned at the species level was <0.01, whereas the K2P distance calculated among the sequences identified only as *Salmo* genus was 0.02.

Mock communities in the fish tanks

The analyses performed on the DNA collected from fish tanks (Table 2, Figure 3) showed that some species can be identified by LAB_A analyses only at the genus level because still missing from the EMBL database (*i.e.*, *Barbus caninus* and *Salmo carpio*) and, for some species, the fragment of the 12S marker amplified by Mifish primers is not informative (see *Oncorhynchus mykiss*, Table S10). More-

Table 1. Species identification of mock samples made of DNA extracted from fish fin clips. For M6, M9 and M14 samples 6, 9 and 14 species were pooled respectively with different proportions of DNA (see Table S1). In bold are highlighted the assignment errors.

		LAB_A analysis			LAB_B analysis		
		INRAE mock samples			INRAE mock samples		
Species present	**Species identified**	**M6**	**M9**	**M14**	**M6**	**M9**	**M14**
Abramis brama (M6, M9, M14)	*Abramis brama*	22926	10147	13607	18950	15261	14656
Ameiurus melas (M14)	*Ameiurus melas*	0	0	0	0	0	648
Coregonus lavaretus (M6, M9, M14)	***Coregonus maraena***	0	0	0	14566	11089	22707
	Coregonus sp.	13315	7380	21001	0	0	0
	Coregoninae	2495	1263	3828	0	0	0
Cyprinus carpio (M9, M14)	*Cyprinus carpio*	0	0	0	0	19272	4010
	Cyprinidae	5452	7880	4378	0	0	0
Esox lucius (M9, M14)	*Esox lucius*	0	18327	0	0	40949	0
	Esox sp.	0	2153	0	0	0	0
Not present	***Leuciscus sp.***	1130	449	675	0	0	0
Perca fluviatilis (M6, M9, M14)	*Perca fluviatilis*	64823	29446	95258	69639	57379	94871
Rutilus rutilus (M14)	*Rutilus rutilus*	**84**	**37**	230	0	0	585
Salmo trutta (M6, M9, M14)	*Salmo trutta*	53479	31158	30666	60915	55216	40149
	Salmo sp.	18439	9415	11116	0	0	0
	Salmo labrax	19	0	15	0	0	0
Salvelinus alpinus (M6, M9, M14)	***Salvelinus fontinalis***	0	0	0	22446	19829	36366
	Salvelinus sp.	32463	17043	44745	0	0	0
	Salmoninae	11735	6261	10338	0	0	0
Silurus glanis (M9, M14)	*Silurus glanis*	0	0	0	0	4231	798
	Silurus sp.	0	56	14	0	0	0
Squalius cephalus (M6, M9, M14)	*Squalius cephalus*	48656	20671	28778	47551	35022	35302
Tinca tinca (M14)	*Tinca tinca*	0	0	399	0	0	991

Table 2. Taxonomic assignment results of the metabarcoding analyses of DNA collected from fish tanks. In bold are highlighted the assignment errors.

		LAB_A analysis	LAB_B analysis
		water tank sample	water tank sample
Species present	**Species identified**		
Barbus caninus	***Barbus ciscaucasicus***	240	0
Carassius carassius	*Cyprinus carpio*	0	1589
	Cyprinidae	891	0
	Engraulis ringens	**70**	0
Lepomis gibbosus	*Lepomis gibbosus*	6394	17451
	Oncorhynchus mykiss	67055	54680
	Oncorhynchus sp.	13361	0
Oncorhynchus mykiss	*Oncorhynchus clarkii henshawi*	**30**	0
	Oncorhynchus nerka	**24**	0
Perca fluviatilis	*Perca fluviatilis*	17376	47373
	Salmo trutta	144596	107995
	Salmo sp.	34515	0
Salmo carpio	*Salmo labrax*	**79**	0
	Salmoninae	3555	0
Tinca tinca	*Tinca tinca*	6059	17533

over, LAB_A analyses detected the presence of *Engraulis ringens*, a species often used to produce a fish meal for aquaculture. All the species present in the fish tank sample were detected by the two laboratories, except *Barbus canius,* which was not identified by LAB_B analyses even at the genus level, and *Carassius carassius*, whose 12S sequence is not taxonomically informative (Table S6). The Venn diagram highlighted that 4 out of 7 species were correctly identified by the two laboratories (Figure 3). The three species missed by the molecular analysis were still absent in the EMBL database or not informative.

Lake Bourget samples

The metabarcoding analyses performed on the environmental samples collected in Lake Bourget showed a good agreement between LAB_A and LAB_B (Figure 4). Only for the rare species represented with a low number of sequences some discrepancies emerged (*e.g.*, *Barbatula bar-*

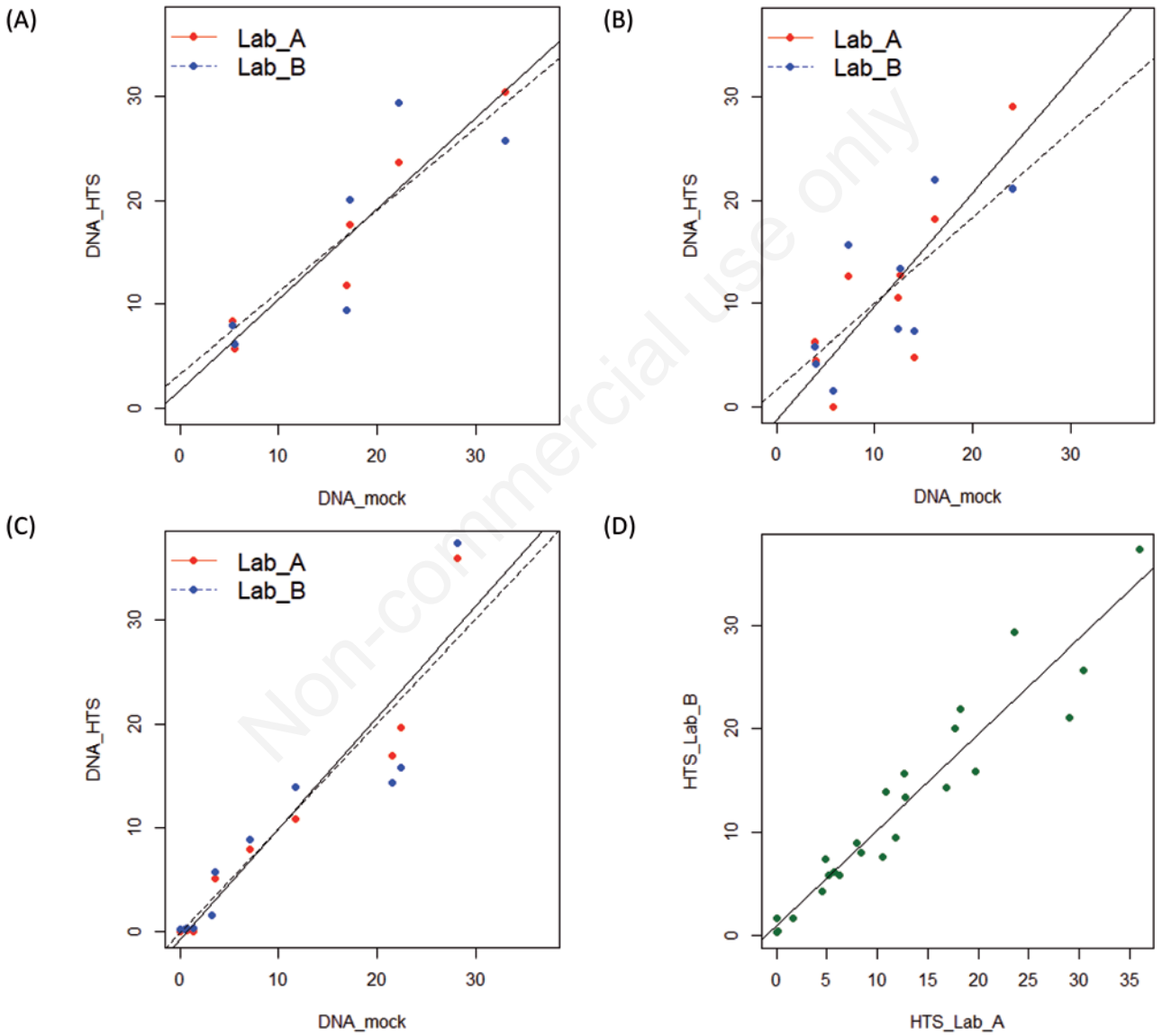

Figure 2. Relationship between the fraction of assigned High Throughput Sequencing (HTS) and the fractions of fish mock DNA pooled in different proportions. Each point represents 1 different species, as represented in Tab 1. The graphs refer to mock assemblages with mix of (**A**) 6, (**B**) 9 and (**C**) 11 species; in (C), three species with a very low amount of DNA were excluded from the analysis; see Tab 1 for details about the species included in the three graphs. In the legend, LAB_A and LAB_B refer to the two sequencing facilities. In the three mock assemblages (graphs A-C), all the regression lines were highly significant ($p<0.001$), whereas both slopes (around 1) and intercepts did not show significant differences. (D) Relationships between the proportions of assigned sequences detected in LAB_A and LAB_B from the analysis of fish mock DNA ($r2=0.92$, $p<0.001$).

batula L., *Gasterosteus aculeatus* L., *Pseudorasbora* sp., *Oncorhynchus mykiss*). The taxonomic identification allowed for the detection of 23 species or genera from the sequences processed by LAB_A, and 20 species from the sequences processed by LAB_B. In total, 13 fish species were detected by both laboratories (without considering the species assigned only at the genus level; Figure 4). As for the other taxa, some species were assigned by Lab_A only at the genus level, and the analyses assigned the sequences to different species within a genus (*e.g.*, *Salmo trutta*).

When comparing the HTS results obtained from the three sampling stations by both LAB_A and LAB_B with the current records of species detected in the whole Lake Bourget by using the traditional sampling approach (mostly electrofishing and gill netting from 1995 to 2018), 17 and 19 species respectively out of 35 were correctly detected (Table 3, Table 4). When considering only the most recent survey, performed in 2018 with traditional gear, 13 out of 15 species were identified by the eDNA metabarcoding approach (Table S11). The regression analyses performed on OTUs numbers, and traditional survey data (fish number and biomass, Table S11) showed significant R values for all the comparisons (Figure S2). The rarefaction curves constructed by using the sequences obtained from Lake Bourget samples showed that the sequencing effort could describe the diversity present in the three samples as all the three curves reached the plateau (Figure S1).

By limiting the comparison only to the assigned sequences identified at the species or genus level by both Lab_A and Lab_B, the results confirmed the high degree of comparability of HTS data (Figure 5). The Spearman correlations between the fractions of OTUs abundances

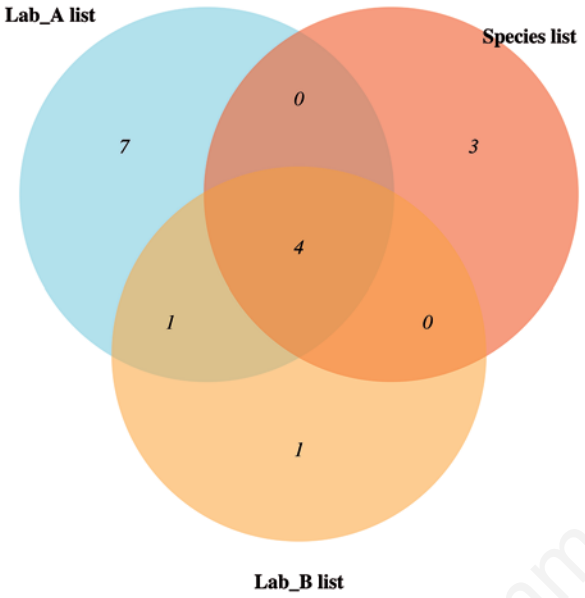

Figure 3. Venn diagram of the eDNA metabarcoding taxonomic identification obtained from the samples collected from the fish tanks at FEM fishery facility. LAB_A = Fondazione Edmund Mach, LAB_B =Nature Metrics.

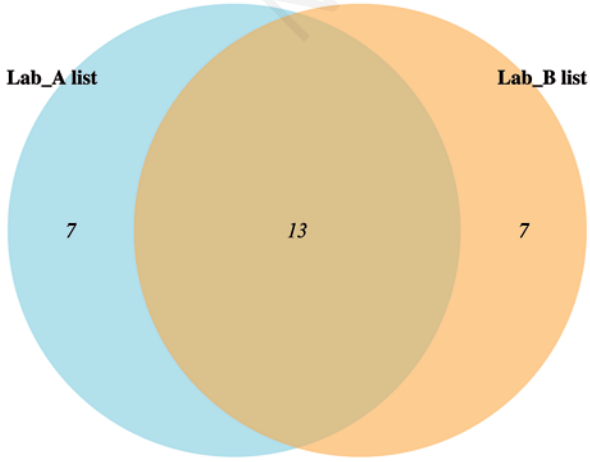

Figure 4. Venn diagram of the eDNA metabarcoding taxonomic identification of the water samples collected in Lake Bourget. LAB_A = Edmund Mach Foundation, LAB_B = Nature Metrics.

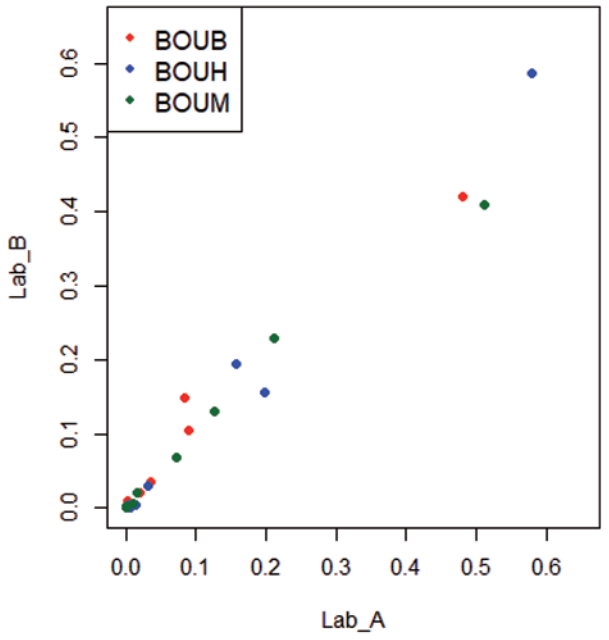

Figure 5. Relationship between the fractions of assigned sequences obtained from the high throughput sequencing (HTS) analyses of the three environmental samples collected in Lake Bourget determined in LAB_A and LAB_B. Each point represents 1 different species. The comparison is limited to the common species found in the two sets of analyses. BouB = low Lake Bourget, BouM = medium Lake Bourget, BouH = high Lake Bourget.

computed on the total of the respective samples obtained in LAB_A and LAB_B for the three sets of samples ranged between 0.84 and 0.85 ($0.001<p<0.03$).

DISCUSSION

In this study, an inter-calibration test has been applied to evaluate the effectiveness of an eDNA metabarcoding protocol in estimating fish community biodiversity in lakes and rivers of the Alpine region. Our results confirmed the need for prior evaluation of the laboratory and bioinformatic protocols by using mock pooled samples of known species composition to validate the bioinformatic procedures, to verify if the protocols used can identify all the species present (and what is the detection threshold) and how reliable the results obtained are. These analyses also allowed to determine the limits of the method and its reproducibility among different laboratories.

Previous surveys have been performed to test the ability to reconstruct the species composition of fish communities using mock samples or aquarium samples (Doi *et al*., 2019; Miya *et al*., 2015; Morey *et al.,* 2020; Thalinger *et al*., 2021b; Turanov and Rutenko, 2020). However, this is the first attempt to reconstruct freshwater fish communities inhabiting the Alpine area using both mock tissue samples and tanks samples.

Table 3. Taxonomic assignment of sequences obtained from the eDNA collected in three different sites in Lake Bourget and analysed by LAB_A and LAB_B metabarcoding protocols. In bold are highlighted the assignment errors.

LAB_A analysis				LAB_B analysis			
Bourget samples				Bourget samples			
Species identified	**BOUB**	**BOUH**	**BOUM**	**Species identified**	**BOUB**	**BOUH**	**BOUM**
Abramis brama	692	0	1093	*Abramis brama*	2953	316	4362
				Ameiurus melas	534	546	0
Barbatula barbatula	0	176	0	*Barbatula barbatula*	612	2302	0
Barbus barbus	256	0	175				
				Cottus gobio	1334	0	0
				Coregonus maraena	3375	1202	8709
Coregonus sp.	4085	2370	18572				
Coregoninae	203	125	2068				
				Cyprinus carpio	70289	307	2349
Cyprinidae	35812	137	1908				
Esox lucius	5843	5005	12986	*Esox lucius*	10326	9827	22440
Esox sp.	364	287	1097				
Esox flaviae	0	0	12				
				Gasterosteus aculeatus	0	244	0
				Gobio gobio	500	0	0
Gymnocephalus cernua	1692	292	0	*Gymnocephalus cernua*	503	419	0
Gymnocephalus sp.	18	0	0				
Lepomis gibbosus	31	795	4123	*Lepomis gibbosus*	890	4199	5216
				Leuciscus leuciscus	6050	892	0
Leuciscus sp.	3400	638	0	*Leuciscus* sp.	901	0	0
Oncorhynchus mykiss	0	0	953				
Oncorhynchus sp.	0	0	26				
Perca fluviatilis	68625	96369	78289	*Perca fluviatilis*	142050	182363	158519
Pseudorasbora sp.	437	0	0				
Rutilus rutilus	17284	25574	43938	*Rutilus rutilus*	26461	62385	65468
Salmo trutta	0	0	0	*Salmo trutta*	78	143	120
Salmo labrax	0	0	896				
Salmo sp.	0	0	44				
Scardinius erythrophthalmus	0	0	414	*Scardinius erythrophthalmus*	794	473	0
				Silurus glanis	462	0	4812
Silurus sp.	41	0	0				
Squalius cephalus	639	429	0	*Squalius cephalus*	2697	0	0
Tinca tinca	24294	31811	25105	*Tinca tinca*	24713	49709	39313

Consistency of high throughput sequencing with the composition of mock and environmental fish assemblages

The pooled DNA analysis extracted from fish fin clips clearly showed that the Mifish primers targeting the 12S rDNA cannot distinguish some species within the *Coregonus*, *Salmo* and *Esox* genus. The amplified fragment is highly conserved in some taxa showing little variation among species/genera, with the result that the taxonomic identification of sequences remains limited to the genus rank. For *Cyprinus carpio,* the BLAST search highlighted the presence of 12S rDNA sequences belonging to different species and genera with a percentage of similarity higher than 97%, which allows an assignment only at the family level. Moreover, some sequences belonging to one individual (*e.g.*, *Salmo trutta* in the mock tissue DNA sample) were assigned to different species or only at the genus level. This finding suggested the presence of some inter-copies variation of 12S rDNA marker within a single individual, as revealed by the K2P distance values, or some remnant PCR/sequencing errors, although a very conservative bioinformatic procedure was used. The analysis performed by LAB_B assigned these identical sequences at the species level but identified, in some

Table 4. Comparison between the fish species recorded using traditional sampling in Lake Bourget (between 1995 and 2018) and eDNA metabarcoding assessment performed by LAB_A and LAB_B. For the species not identified by the metabarcoding approach, we have checked the presence of 12S rDNA MiFish fragments in EMBL database: 0 = missing, 1 = present.

	Traditional sampling recordseDNA metabarcoding LAB_AeDNA metabarcoding LAB_BPresence of reference sequence				
	Species	**Note**	**Taxonomic assignment**	**Taxonomic assignment**	**12S MiFish fragment**
1	*Abramis brama*		*Abramis brama*	*Abramis brama*	
2	*Alburnoides bipunctatus*				0
3	*Alburnus alburnus*				1
4	*Ameiurus melas*			*Ameiurus melas*	
5	*Barbatula barbatula*		*Barbatula barbatula*	*Barbatula barbatula*	
6	*Barbus barbus*		*Barbus barbus*		
7	*Blicca bjoerkna*				1
8	*Coregonus lavaretus*		*Coregonus* sp.	*Coregonus maraena*	
9	*Cottus gobio*			*Cottus gobio*	
10	*Cyprinus carpio*			*Cyprinus carpio*	
11	*Esox lucius*		*Esox lucius*	*Esox lucius*	
12	*Gasterosteus aculeatus*			*Gasterosteus aculeatus*	
13	*Gobio gobio*			*Gobio gobio*	
14	*Gymnocephalus cernua*		*Gymnocephalus cernua*	*Gymnocephalus cernua*	
15	*Lampetra planeri*				0
16	*Lepomis gibbosus*		*Lepomis gibbosus*	*Lepomis gibbosus*	
17	*Leuciscus leuciscus*		*Leuciscus* sp.	*Leuciscus leuciscus*	
18	*Lota lota*				1
19	*Oncorhynchus mykiss*		*Oncorhynchus mykiss*		
20	*Perca fluviatilis*		*Perca fluviatilis*	*Perca fluviatilis*	
21	*Phoxinus phoxinus*				1
22	*Pseudorasbora parva*		*Pseudorasbora* sp.		
23	*Rhodeus amarus*				1
24	*Rutilus rutilus*		*Rutilus rutilus*	*Rutilus rutilus*	
25	*Salaria fluviatilis*				0
26	*Salmo trutta*		*Salmo* sp.	*Salmo trutta*	
27	*Salvelinus alpinus*				1
28	*Salvelinus fontinalis*	*only once in 1995 and uncertain*			1
29	*Sander lucioperca*				0
30	*Scardinius erythrophthalmus*		*Scardinius erythrophthalmus*	*Scardinius erythrophthalmus*	
31	*Silurus glanis*		*Silurus* sp.	*Silurus glanis*	
32	*Squalius cephalus*		*Squalius cephalus*	*Squalius cephalus*	
33	*Telestes souffia*				0
34	*Thymallus thymallus*				1
35	*Tinca tinca*		*Tinca tinca*	*Tinca tinca*	

cases, a wrong species (*e.g.*, *Coregonus maraena* and *Salvelinus fontinalis*). In general, the two laboratories were able to describe the fish community composition at least at the genus level. Only three species, namely *Gobio gobio*, *Esox lucius* and *Lota lota*, which were present in very low proportions of DNA (<0.01%, Table S1), escaped the identification.

The fish tank experiments were consistent with the above results, highlighting a good performance of the metabarcoding analyses with some limits due to the absence of reference sequences in the database or the lack of resolution of the fragment of 12S rDNA used for the taxonomic identification. Also, in this analysis, the presence of intra-individual 12S rDNA copy variation emerged for *Salmo trutta* and was suggested for *Oncorhynchus mykiss* as a very low number of sequences were assigned to two different species (*Oncorhynchus clarkii henshawi* and *Oncorhynchus nerka*) and an important number of sequences were assigned at the genus level. In fish tanks, several individuals per species were present and, for this reason, the computation of the intra-individual 12S rDNA copy variation was not feasible. The identification of a low number of sequences misassigned for *Salmo* and *Oncorhynchus* suggested that these copy variants could be present in low frequency in the mitochondrial genome of these species. Interestingly, LAB_A detected a species commonly used as fish meal, namely *Engraulis ringens,* which was probably present only in traces as very few sequences were detected. This outcome confirms the high sensitivity of this method, which can identify traces of DNA in a pool of more abundant genetic material, as also revealed by the results obtained from the tissue DNA mock sample.

The analyses of "real" environmental samples collected in Lake Bourget confirmed the results of mock samples showing that both LAB_A and LAB_B approaches provided a comparable description of the fish community in the lake. Some discrepancies emerged for a few species that showed a low number of sequences and was probably represented by a very low amount of DNA. Our test on the tissue DNA mock sample showed a threshold level of detection as low as 0.05% (Table 1 *Rutilus rutilus*). The taxonomic assignment of *Salmo* sequences highlighted that LAB_A detected only *Salmo labrax* or *Salmo* sequences, whereas LAB_B identified *Salmo trutta* in the same sample. *Salmo labrax* was never recorded in Lake Bourget, and is present only in the Danube area and Eurasia rivers draining to the Black Sea. By inspecting these sequences, it appears that *Salmo trutta* and *Salmo labrax* sequences differ only for one nucleotide change out of a total of 170 nucleotides of the 12S rDNA fragment. This confirms that special attention should be given when considering the taxonomic assignment of sequences belonging to these species and further inspection (also using a BLAST search) is advisable. Notably, the three 6 x 2 L water samples collected in the lake were able to detect 17 and 19 species (LAB_A and LAB_B, respectively) out of 35 species recorded from 1995 to 2018 through the classical collection and identification methods, and the sequencing effort used revealed to be able to identify all the species present in the samples. This outcome stresses the high potential of this technique for the description of fish biodiversity in lakes and rivers, despite some limits highlighted by this intercalibration test. Issues implicit in the taxonomic resolution of the genetic markers should be carefully evaluated and, in this regard, a further taxonomic assignment step using a customised database, including only the target species already recorded in the habitat under study, can help improve the assignment of sequences at the species level (as shown by LAB_B results).

A few species were not identified because they were not present in the reference databases (Table 4). A more comprehensive database for the taxonomic assignment is recommended to detect allochthonous species that would not be included in the customised database of the selected species. Moreover, a known limit of the eDNA approach (and barcoding approach in the broader sense) is the lack of information in public databases that can prevent the identification of some species which can be undetected. A further obvious control of the reliability of the species identified by using the eDNA approach is the plausibility of the taxonomic composition of the fish community described using *a priori* information of the ecosystem under study. This information can be obtained for lakes and rivers of the Alpine region from the systematic assessment performed by using the classical sampling gears as in the case of Lake Bourget, described in this work.

Quantitative assessment of fish communities

Besides testing the efficiency in detecting single species, the experiments carried out with the tissue DNA mock samples allowed us to verify that the potential PCR and sequencing errors do not influence the final proportion of DNA sequences assigned for each species. In this regard, the experiments were based on controlled conditions and analyses carried out on predetermined fractions of pure DNA extracted from single specimens, therefore removing all the biases that usually are present when "blind" comparisons, without previous knowledge of communities, are carried out in the evaluation of laboratory performances. The HTS in the LAB_A and LAB_B identified the sequences associated with the respective fish species with almost the same proportions as those used to artificially create the three mock samples. Further, the two laboratories showed comparable

performances, indicating a good reproducibility of methods. As for this last aspect, and limiting the analyses to the only common species found in the two laboratories, a good reproducibility was obtained by comparing the fractions of the sequence abundances of the species identified taxonomically in the LAB_A and LAB_B.

The above results highlight and further demonstrate the sensitivity of the HTS methods in quantifying the number of specific gene markers in heterogeneous genomic DNA extracts, although several issues intrinsic to the procedure only allow for a relative estimation of fish biomasses. These include the sample collection and the nature and stochastic distribution of biological tissues and materials released by fish (Jo *et al*., 2019; Sassoubre *et al.,* 2016). Many environmental parameters, like mixing/dispersion and the time spent by fish in the area, as well as biotic parameters (skin drop off rates and DNA decay rates), are indeed important factors to relate eDNA concentrations to actual fish biomass and abundance. Previous studies reported a temperature-dependent degradation of eDNA (Eichmiller *et al.*, 2016; Lance *et al*., 2017; Strickler *et al*., 2015; Tsuji *et al.*, 2017), an accelerated eDNA degradation due to higher water temperatures and higher fish biomass (Jo *et al*. 2019) and a correlation with physiology and behaviour of different species groups (Thalinger *et al*. 2021b).

CONCLUSIONS

The eDNA HTS approach to monitor fish biodiversity in Alpine lakes and rivers proved to be an efficient tool to complement the current biomonitoring surveys, and the correct implementation of the metabarcoding method and interpretation of the results obtained is crucial to avoid wrong conclusions.

This work demonstrated a good comparability of the two laboratories participating in the inter-calibration test results. In particular, the differences in the quantitative estimation of the relative abundances of OTUs in three fish mock assemblages were insignificant. On the other side, a few relevant differences were apparent in the discrimination and taxonomic annotation at the species or genus level of selected taxa. Possibly, one of the most serious issues in the application of HTS methods is still represented by the incomplete coverage of species in the nucleotide databases. This has important implications when HTS methods are used in the biomonitoring of aquatic environments or for the early detection of non-indigenous species. These drawbacks should be taken into consideration when interpreting eDNA data. In the case of biomonitoring, it is urgent to complete the coverage of reference databases, at least on a regional basis, including not only native species, but also those recognised as potential non-indigenous species.

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