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Detection of the Endangered Stone Crayfish *Austropotamobius torrentium* (Schrank, 1803) and Its Congeneric *A. pallipes* in Its Last Italian Biotope by eDNA Analysis

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Abstract: The stone crayfish, *Austropotamobius torrentium*, is a European freshwater crayfish. Although this species is relatively widespread throughout the continent, it is undergoing significant declines throughout its range. However, as the decline rates have not been quantified in detail, this species is classified as data deficient by the IUCN Red List of Threatened Species. The present study describes the development and validation of two species-specific assays based on hydrolysis probe chemistry for the detection of *A. torrentium* and *A. pallipes* environmental DNA (eDNA) in water samples collected in the Julian Alps of Italy (Friuli Venezia Giulia). The eDNA-based method was applied to 14 sites within the Danubian Slizza basin, known to be inhabited by *A. torrentium*, but with insufficient information on their distribution. In addition, one station in the Tagliamento River basin was sampled to test the performance of the *A. pallipes* probe. The presence of *A. torrentium* is confirmed at 6 out of 15 sites. At four of these sites, *A. torrentium* is detected for the first time. In contrast, the presence of *A. torrentium* was not detected at two sites already known to harbour the species. Finally, the presence of *A. pallipes* was confirmed in the station belonging to the Tagliamento basin. The methodology described, which allows the distinction between the two species, paves the way for the parallel detection of the stone crayfish and the white-clawed crayfish (*A. pallipes*) through eDNA analysis.

Keywords: *Austropotamobius torrentium*; *Austropotamobius pallipes*; detection; conservation; environmental DNA; eDNA; crayfish monitoring; 16S rRNA

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1. Introduction

The stone crayfish, *Austropotamobius torrentium* (Schrank, 1803), is a freshwater species native to Central and South-eastern Europe [1–3]. The species inhabits mainly small, cold-flowing waters at mid-to-high elevations, characterised by moderate gradient, high hydromorphological and bed heterogeneity, and abundant riparian vegetation [4].

Habitat loss, primarily due to channel modification (e.g., concrete revetment), agriculture, pollution, and exotic species that act as both competitors and parasitic disease vectors, threaten the survival of *A. torrentium* [5,6]. The risk of extinction is further increased by its slow life-cycle with low fecundity and high habitat specialization [2,4,7,8]. Moreover, like other European crayfish, it is also susceptible to crayfish plague [9–12].

A. torrentium is listed as a "data deficient" (DD) species in the IUCN Red List of Threatened Species [13]. It is also protected internationally by the Bern Convention (Appendix III, protected fauna species) and as a species of community interest on the territory of the

European Union by the EU Habitats Directive 92/43/EEC, where it is listed in Annex II (core areas of the species' habitat are designated as Sites of Community Importance (SCIs) and included in the Natura 2000 network) and in Annex V (member states can decide how to manage the population, but must ensure that their exploitation and collection from the wild is compatible with maintaining a favourable conservation status). Furthermore, this species is protected by national legislation throughout most of its range [13,14]. As most of the natural range of *A. torrentium* lies within the territory of the EU, the Union has a particular responsibility for the conservation of this species. It considers it a priority species for conservation—highlighted by an asterisk (*) before the species name in the Annex II. In addition, this crayfish is protected by national laws in most parts of its range [14,15]. As *A. torrentium* has been placed under EU legal protection by the Habitat Directive, Member States are committed to maintain and restore the so-called "favourable conservation status" of this species and regularly monitor and report on the conservation status and trends to the European Commission [16]. Furthermore, the rate of decline of the stone crayfish is unknown in a large part of its range. Therefore, measures to assess the status of its populations and map its distribution in detail are urgently needed.

In Italy, the distribution of *A. torrentium* is restricted to the Slizza basin in the surroundings of Tarvisio in the north-easternmost part of the country (Udine Province, Friuli Venezia Giulia, FVG, Italy), where extensive field surveys have identified four sites, all harbouring small populations, and one site where the species has likely become extinct [17–19]. The Slizza is an alpine torrent in Italy and Austria that originates below the Sella Nevea Pass in the Julian Alps and flows into the Zilja (a right tributary of the Drava) in Carinthia, Austria. In addition to the Slizza, the Danubian watershed also marginally penetrates Italy via some tributaries of the Inn and a single tributary of the Sava, but no *A. torrentium* has ever been recorded here.

In the Habitats Directive reports to the EU Commission, the conservation status of the species was classified as unfavourable-bad (U2) for the period 2007–2012 [20], and for the period 2013–2018 the conservation status of the species could not be evaluated. As no records of the species occurrence were made during the reporting period, it was not possible to assess whether it occurs regularly and in significant numbers in the region. Furthermore, the Italian Ministry of Environment, Land and Sea Conservation described the information on *A. torrentium*, as "data deficient" (Prot. 0017966 PNM of 17 September 2015). As an outcome, the occurrence of the species was classified as uncertain or with Scientific Reserve (SCR).

Environmental DNA (eDNA) analysis has become an established tool for ecologists, particularly for monitoring rare, cryptic and invasive species or for those species where traditional methods may be ineffective, labour-intensive, or detrimental [21,22]. eDNA has been used in a variety of studies focused on monitoring various freshwater animals from lotic, lentic and even subterranean habitats [23–29]. Rather than sampling organisms directly, this method also allows species to be traced through the detection of genetic material scattered in the environment, usually in the form of moulting remains, urine and faeces, mucus and gametes.

Recently, eDNA has been successfully used to monitor the distribution of *Austropotamobius pallipes* (Lereboullet, 1858) in the British Isles [30,31], in Switzerland [32], and in Germany [33]. The species has been detected using a species-specific qPCR assay targeting the mitochondrial gene coding the cytochrome oxidase I (COI) protein. Studies show that eDNA can be detected within hours of the animal's arrival [34], although it usually takes days or even weeks for eDNA to be detected when biomass is low [29,35]. Reports of limit-of-detection (LOD) for smaller aquatic animals with eDNA qPCR assays are around one organism per several hundred m^3 and are reasonably consistent across a range of environments, from subterranean to tropical [29,36]. This suggests that eDNA results can provide a realistic picture of the occurrence of the target species. Monitoring freshwater crayfish using traditional methods such as traps is effective but time-consuming [37,38]. In addition, traps and nets serve to act as vectors for crayfish plague [12,39].

In this manuscript, we present and evaluate the performance of two assays based on specific primers and hydrolysis probes targeting a variable region of 16S rRNA, that can discriminate between *A. torrentium* and *A. pallipes* complex. Both assays were first evaluated in silico with most sequences of *A. torrentium* and *A. pallipes* deposited in GenBank and in an in-house reference database, as well as with DNA from tissue samples of native and non-native freshwater crayfish in the FVG and adjacent Slovenia; these samples were also used to test for cross-amplification. Finally, the assays were tested in a study on eDNA samples collected in the Slizza basin in the FVG, Italy, to determine a preliminary distribution of *A. torrentium* in Italy.

2. Materials and Methods

2.1. Study Design

The eDNA methodology for the detection of *A. torrentium* and *A. pallipes* in streams was developed primarily to clarify the SCR classification of *A. torrentium* before the next Habitats Directive reporting to the EU Commission (2019–2024), and to study the distribution of *A. torrentium* in the FVG, the only Italian region where both species occur and for which the latest information dated back to 2015 [20]. The development of this methodology involved the following steps: (i) development of two species-specific assays based on probe hydrolysis allowing the detection of *A. torrentium* and *A. pallipes* eDNA; (ii) testing the specificity of both assays on genomic DNA (gDNA) of the two target species and other crayfish present or potentially present in the FVG; (iii) testing the LOD of the method under laboratory conditions; and (iv) testing the performance of the assays in a pilot study on water samples collected in the wild.

2.2. Primers Design and Species-Specificity Tests

Conservative regions in the 16S rRNA sequences suitable for designing specific primers and probes for *A. torrentium* and *A. pallipes* were identified from alignments and two consensus sequences built with MEGA X [40] and CLC Genomics Workbench v21 (Qiagen, Hilden, Germany). *A. torrentium* alignment included 49 sequences deposited in GenBank and sequences of two specimens collected in the "Rutte piccolo" sampling point, near Tarvisio during this study (Supplementary Material File S1A); *A. pallipes* alignment included 567 sequences deposited in GenBank and sequences from 428 specimens collected during 2011 and 2013 from 63 sampling sites spread throughout FVG (Supplementary Material File S1B) [41]. The query used to search the GenBank was "16S" and "*Austropotamobius torrentium*" or "16S" and "*Austropotamobius pallipes*" for the two species. Primers and probes were designed using Primer3Plus v. 4.0.0 [42], while the presence of dimers and hairpin formations were checked with OligoCalc v. 3.27 [43]. Possible cross-amplification between the two species and non-native crayfish present in FVG and neighbouring areas was first evaluated in silico (Supplementary Material File S1C). MEGA X [40] was used to compare the designed primers and probes with Blast searches against *A. torrentium* or *A. pallipes* and against *Procambarus clarkii* (Girard, 1852) and *Faxonius limosus* (Rafinesque, 1817) 16S rRNA sequences deposited in GeneBank, for a total of 41 sequences for these two invasive crayfish (Supplementary Material File S1C). *P. clarkii* is present both in FVG [44] and in neighbouring Slovenia [45], while *F. limosus* is currently only present in Slovenia [46]. The universality of the two assays was investigated by a phylogenetic network approach. Regions corresponding to the primers and probes were extracted and concatenated to build subsets of the two alignments and the median-joining algorithm ($\varepsilon = 0$; [47]), as implemented in the PopART software v 1.7.2 [48], was used to construct phylogenetic networks. In addition, similarity between *A. torrentium* assay (hereinafter *Ator*) and *A. pallipes* (hereinafter *Apal*) and vice versa were evaluated by using each of the two assays as an outgroup to the other alignment.

2.3. Animals Sampling and Genomics DNA Analysis

The species-specificity of the primers was also tested on genomic DNA (gDNA) extracted from tissue samples of *A. torrentium*, *A. pallipes*, *P. clarkii* and *F. limosus* (2, 10, 4

and 1 specimen, respectively) collected in FVG and Slovenia. Genomic DNA was extracted with E.Z.N.A.® Mollusc DNA Kit (Omega Bio-Tek, Norcross, GA, USA) following the manufacturer's instructions. Extracted DNA was checked for quality and concentration with a NanoDrop™ 2000 Spectrophotometer (Thermo Scientific; Thermo Fisher Scientific Inc., Waltham, MA, USA).

Field morphological species identification (including the claws remains collected near Station 2) was verified with DNA barcoding of the COI with primers LCO-1490 and HCO-2198 [49]. DNA was amplified with 1 U KAPA HiFi HotStart DNA Polymerase (KapaBiosystems, Wilmington, MA, USA), 1X KAPA HiFi Buffer, 20 ng DNA, 0.3 µM for both forward and reverse primers, 0.3 mM/each deoxynucleotides (dNTPs), and PCR-grade water up to a final volume of 15 µL. PCRs were performed in the Mastercycler ep Gradient S thermal cycler (Eppendorf, Hamburg, Germany) under the following conditions: initial denaturation at 95 °C for 3 min, 10 cycles at 98 °C for 20 s, 45 °C for 15 s and 72 °C for 45 s, 30 cycles at 98 °C for 20 s, 54 °C for 15 s and 72 °C for 45 s, and a final elongation step at 72 °C for 2 min. The PCR products were sequenced using BigDye 3.1 sequencing chemistry (Applied Biosystems, Waltham, MA, USA) and analysed on an ABI3130 capillary sequencer (Applied Biosystems). Species identification was validated by a BLASTn similarity search [50] using the GenBank database.

DNA extracted from the native and non-native crayfish (4 and 3 specimens, respectively) was also used to test cross-amplification of the two assays. Reaction mix contained 20 ng of gDNA, 1X KAPA Probe Force qPCR Mastermix (code# KK4300, Sigma-Aldrich, Saint Louis, MO, USA), 0.2 µM for both forward and reverse primers, 0.1 µM specific probe, and PCR-grade water up to a final volume of 15 µL. qPCR analysis was performed in the Bio-Rad CFX 96 Real time System (Bio-Rad Laboratories, Inc., Hercules, CA, USA) under the following conditions: initial denaturation at 98 °C for 3 min, 50 cycles at 95 °C for 10 s and 64 °C for 25 s. In addition, *A. torrentium* and *A. pallipes* gDNA were used as positive control samples in the downstream analysis.

2.4. Environmental DNA Sampling and Analysis

Sampling activities were performed in the summer (July and August 2017, and July 2018) and fall (October 2017). The sampling area was constrained to the Slizza basin (14 sites) except for a single station in the Tagliamento basin (Station 15, geographical coordinates 46.494 N, 13.600 E). The sites sampled in this study are all at about 730 to 930 m above sea level (m.a.s.l.), except Station 15 which is at about 450 m.a.s.l.

Due to the risk of poaching, and considering the small estimated size of *A. torrentium* populations in Italy [20], exact geographical coordinates are not reported but are available upon consultation with the authors.

One litre of water was filtered directly at each sampling site with Sterivex durapore PVDF 0.45 µm filters (Merck-Millipore, Burlington, MA, USA) using a sterile disposable syringe of 50 mL (BDPlastipak™, Franklin Lakes, NJ, USA). Each filter was wrapped in at least two bags and delivered refrigerated to the laboratory at the Department of Life Sciences (University of Trieste, Trieste, Italy), where it was stored at −20°C until eDNA extraction. DNeasy PowerWater Sterivex kit (Qiagen) was used to extract eDNA according to the manufacturer's guidelines.

To avoid type I errors (false positives), standard operating procedures for eDNA, including UV decontamination of the pipettes, exclusive use of filtered pipette tips, NaClO 4% solution and UV decontamination of surfaces, use of reagent aliquots, frequent changing of gloves, and inclusion of Negative Template Control (NTC) and Positive Template Control (PTC) in eDNA extraction and qPCR analysis have been adopted rigorously, along with physical separation of areas where eDNA extraction, PCR preparation and post-PCR steps were taking place.

For the qPCR analysis, the same reaction mix and thermal profile were used as for the cross-contamination tests, except that 2 µL of eDNA were used instead of 1 µL per reaction.

LOD [51] and inhibition tests [52] were evaluated preliminarily with details provided in the Supplementary Material, (Files S7.1 and S8.1, Figures S7.2 and S8.2).

eDNAs were analysed in four technical replicates, while in some cases, five replicates were used (File S9). Each run included PTC and NTC. PTC consisted of 1 µL (~20 ng) of gDNA extracted from *A. torrentium* and *A. pallipes* samples, while NTC included ultrapure water. eDNA samples were considered as positive when a sigmoidal signal was observed in at least two qPCR technical replicates and uncertain when it was observed only in a single technical replicate; amplification curves that crossed the common fluorescence threshold within 40 cycles and had a sigmoidal shape were considered as observed signals [53].

Some amplicons from eDNAs were also sent to an external service for Sanger sequencing (Eurofins, Hamburg, Germany) to confirm PCR detection.

3. Results

3.1. Apal and Ator Assays Species-Specificity

The specificity of *A. torrentium* assay (*Ator*) was first evaluated by aligning it against all *A. torrentium* 16S rRNA sequences available from GenBank (49 sequences downloaded on 15 May 2020), plus two local *A. torrentium* sequences of *A. torrentium* (GenBank OM422805 and OM422806). Sequence GQ168827, downloaded from GenBank, was excluded as it did not overlap with the target area of the primers, thus a total of 50 sequences were included in this evaluation (Figure 1A).

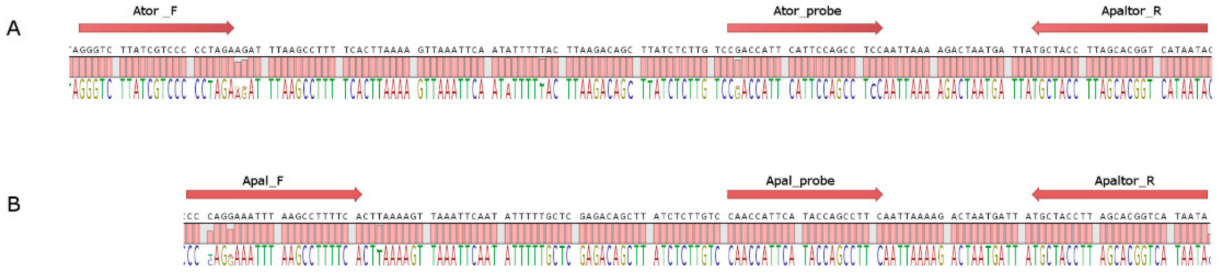

Figure 1. Alignments displayed as consensus sequences with logo representations of the sequence conservation. (**A**) The consensus alignment of 50 *A. torrentium* (*Ator*) sequences and (**B**) the consensus alignment of 996 sequences from *A. pallipes* (*Apal*). Red arrows indicate oligonucleotides positions.

The specificity of *A. pallipes* assay (*Apal*) was tested in the similar fashion, against all the 16S rRNA *A. pallipes* sequences from *A. pallipes* available from GenBank (567 sequences downloaded on 15 May 2020), plus 429 local *A. pallipes* sequences [41]. A total of 996 sequences were included in this evaluation (Figure 1B).

The *Apal* and *Ator* assays shared a common reverse primer (*Apaltor_R*), but both had a specific forward primer and an oligonucleotide probe, which were designed to discriminate between the *A. pallipes* and *A. torrentium* sequences (see Table 1).

Table 1. *Apal* and *Ator* primer sequences and the size of the amplicon produced.

Target Species	Sequence ID	5′-3′ Sequence	Amplicon Size (Base Pairs)
A. torrentium	*Ator_F*	GGGTCTTATCGTCCCCCTAGA	152 bp
	Ator_probe	[FAM]CGACCATTCATTCCAGCCTCC[BHQ1]	
A. pallipes	*Apal_F*	CCCAGGAAATTTAAGCCTTTTCA	136 bp
	Apal_probe	[FAM]CAACCATTCATACCAGCCTTC[MGBEQ]	
In common	*Apaltor_R*	TATTATGACCGTGCTAAGGTAGCA	

To additionally check the species-specificity of the assays produced, network analysis was performed using a median-joining algorithm. Tailored sequences for both *A. torrentium* and *A. pallipes* groups were built selecting exclusively the part overlapping primers and probes. Figure 2A shows the network obtained for the *Ator* assay (the list of the sequences

used in the *Ator* network are provided as Supplementary Material File S3). Figure 3A shows alignment of the unique haplotypes to *Ator* assay. The node identified as AM181348 includes the sequences fully matching our *Ator* assay. The JF293373 node represented a single specimen from Croatia that possesses a single mismatch (C/T) at position 18. The node JF293369 included five sequences, four from Croatia and one from Bulgaria (JF293380); this node represents one (A/G) mismatch at position 23 of the alignment, corresponding to the second nucleotide of the probe. A third node (JF293383) includes three sequences from Croatia with two mismatches, a first identical to the cluster JF293369 and a second one corresponding to T/C at position 41 in the alignment and at the 20th nucleotide of the *Ator_probe*.

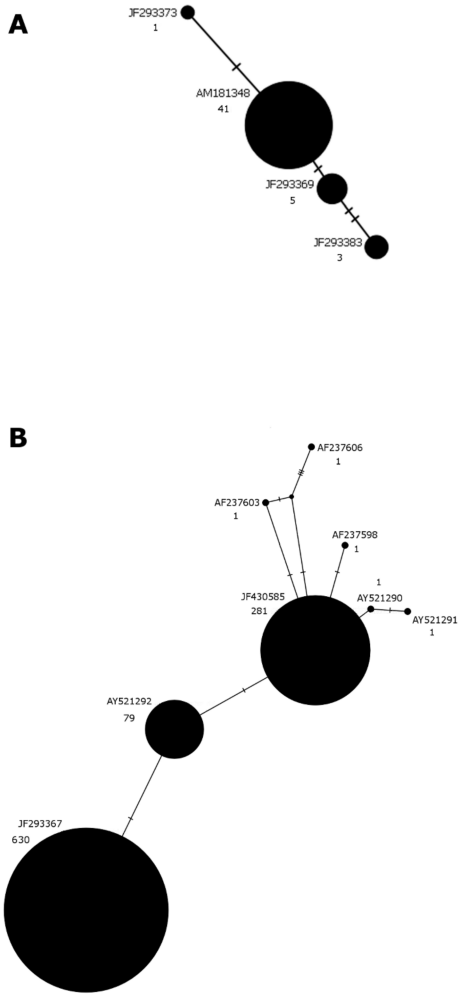

Figure 2. (**A**) Haplotype network for the *Ator* assay and (**B**) for the *Apal* assay. Haplotype circle dimensions are proportional to the number of the sequences belonging to each cluster, numbers are specified nearby each node name. Edge lengths are equal to pairwise distances between sequences and hatch marks alongside the branches indicate the number of mutations occurring to the reference assay. The complete list of the sequences for each node is provided as Supplementary Material Files S3 and S4 for *A. torrentium* and *A. pallipes*, respectively.

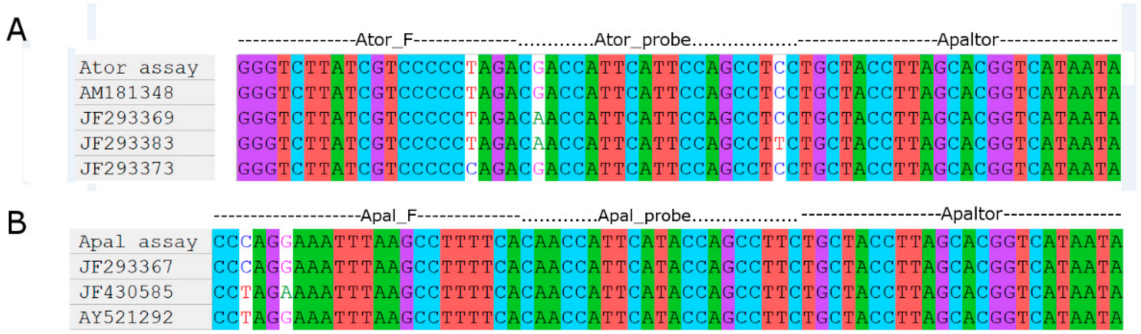

Figure 3. (**A**) Alignment of the unique haplotypes shown in Figure 2A compared to their species-specific *Ator* assay; (**B**) alignment of the unique haplotypes shown in Figure 2B compared to their species-specific *Apal* assay. The nucleotides highlighted in white are not 100% conserved in all aligned sequences.

For the *Apal* assay, an initial analysis of local sequences [41] was assessed by a network analysis, revealing 17 different local haplotypes (Figure S2). Similarly, also for the *Apal* assay, a network analysis was run on all the sequences downloaded from the GenBank or collected locally in the FVG [41], in total 996 sequences. Following the default parameters of the median-joining algorithm with epsilon set at 0, five sequences were masked because they contained >5% of undefined sites (Figure 2B and Supplementary Material File S4).

The cluster identified as JF293367 (Figures 2B and 3B) corresponds to the 63.6% of the 16S rRNA from *A. pallipes* stored at GenBank and 95% of the local sequences, so it includes all the local haplotypes except hap6 sequences (GenBank OM422812). The distribution of the non-local sequences included in this cluster is variable and covers Italy, Croatia, Bosnia and Herzegovina, Montenegro and France. The node AY521292 includes 23 local sequences from *A. pallipes* belonging to the haplotype 6, which presents a single T/C mismatch corresponding at the second nucleotide of the *Apal_F*. The remaining sequences (280) presented more than two mismatches variably distributed on the considered fragment, mostly on the *Apal_F* primer.

Species-specificity of each assay was additionally verified by a haplotype network analysis done on *A. torrentium* sequences compared with the *Apal* assay, which did not cluster with any of the stone crayfish samples, showing the presence of at least 16 mismatches with the sequences used (Figure S5, Supplementary Material) and on *A. pallipes* sequences versus *Ator* assay, and also in this case *Ator* did not cluster with any *A. pallipes* sequencing due to the presence of 15 mismatches (Figure S6, Supplementary Material).

Species-specificity tests were performed on different biological samples, PCRs were run on gDNA from *A. torrentium*, *A. pallipes*, *P. clarkii*, and *F. limosus*. All sets of primer and probes amplified target species, and did not cross-amplify the invasive decapod species.

3.2. Results from Local Samples

The positive hits from eDNA results are shown in Figures 4 and 5, and the overall results are provided in detail in File 9 (Supplementary Material) alongside the Ct obtained for each replicate.

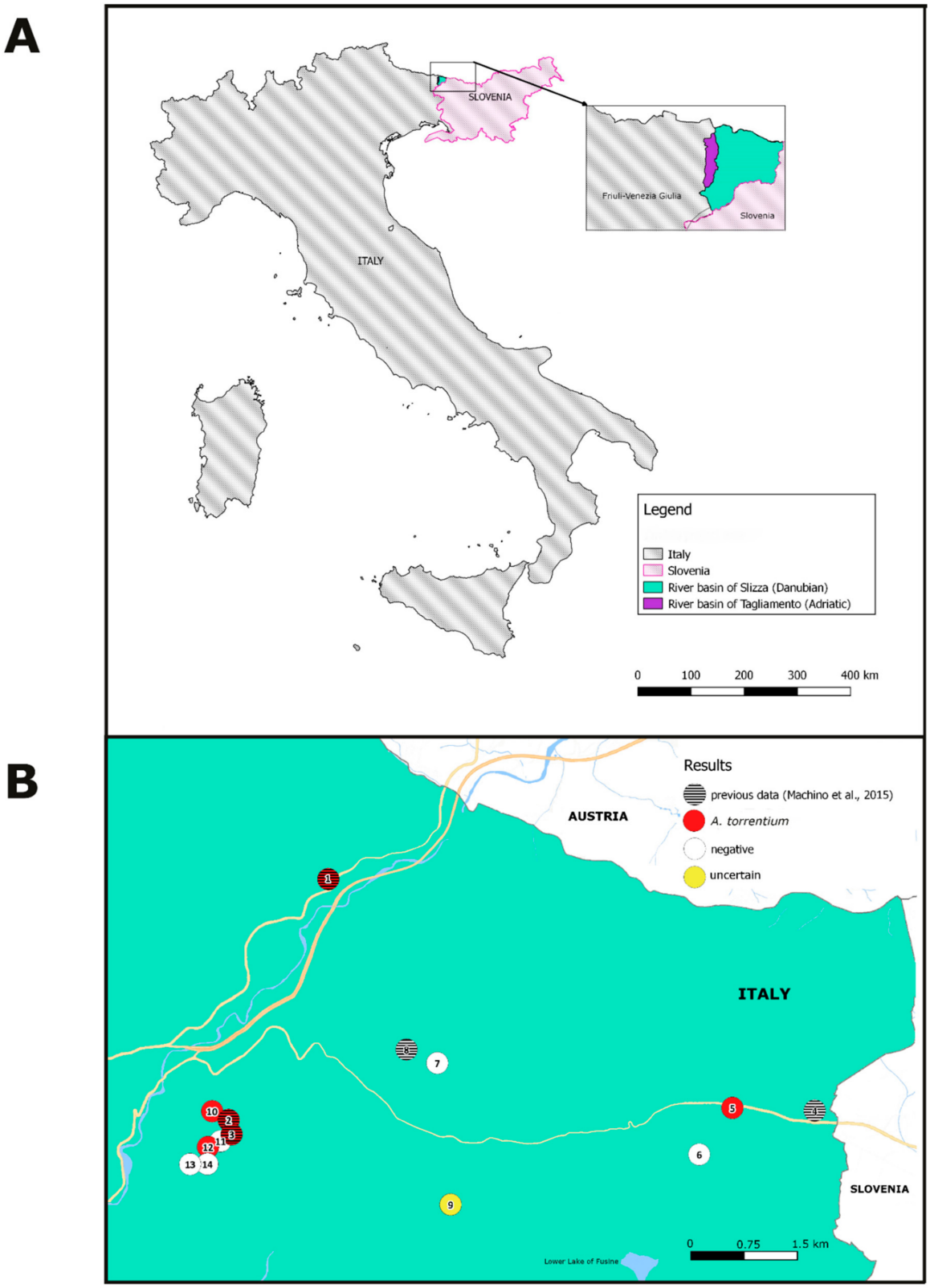

Figure 4. (**A**) Geographical location and map of the target area reported in green and purple based on the basin belonging within. (**B**) Map of the 14 sampled stations from the Slizza basin (Station 15 from the Tagliamento basin, reported in purple in section A is not shown, but geographical coordinates are given in Section 2.4).

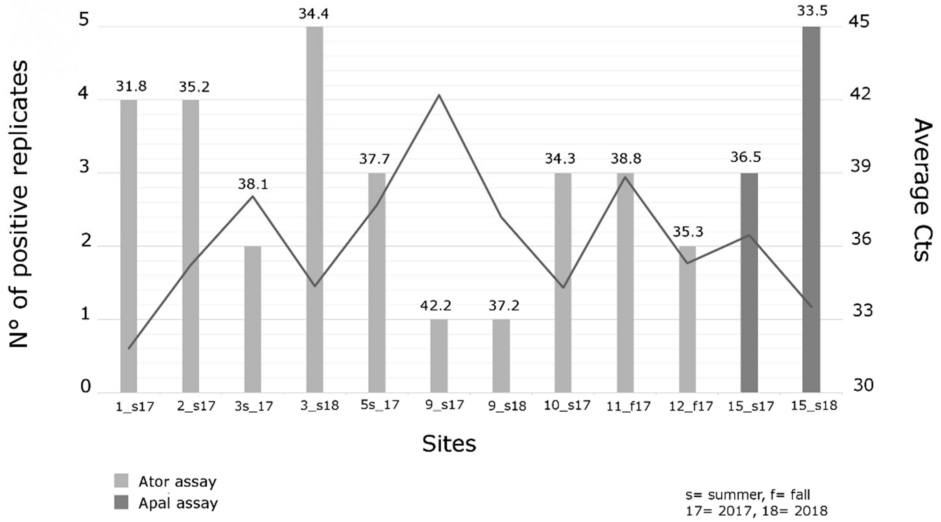

Figure 5. Positive amplifications obtained in eDNA samples by qPCR alongside their average cycle thresholds (Cts) are shown as a solid line or as a number on the top of each bar. The number on the x-axis indicates the station, s (= summer) and f (= fall) stand for the season when each eDNA sample was taken, and 17 or 18 indicates the year of collection. Light grey indicates the positive replicates obtained with the *Ator* assay and dark grey indicates the amplifications achieved with the *Apal* assay.

Stations 1–3 were confirmed as sites inhabited by the *A. torrentium* populations, as already reported in Machino et al. [19]. The presence of *A. torrentium* within Station 2 was also confirmed by Sanger sequencing of a claw found nearby the sampling site. This sequence was very similar (99.65%) to the COI haplotype 9 [54] that belongs to the CSE phylogroup, and to sequences from neighbouring Slovenia (e.g., AY667127 and AM180947). Stations 4 and 8, previously reported by Machino as sites with *A. torrentium*, scored negative for stone crayfish presence; Station 4 was tested for two consecutive years. However, *A. torrentium* has been "eDNA detected" at a nearby Station 5 located one kilometre westward from Station 4 (Figure 3 and Supplementary Material File S9). Stations 10–12, alongside the already mentioned Station 5, represent new locations with confirmed presence of *A. torrentium*. One site remains "uncertain", Station 9, which in one summer (2018) scored a positive Ct value for *A. torrentium* in 1 out of 5 replicates. Site 15, the only station falling in the Tagliamento basin, scored positive for *A. pallipes* presence both in fall and summer seasons, and as expected, no detection of *A. torrentium* was scored during the monitoring. The remaining sites did not show any detections.

4. Discussion

This study provides the most complete up-to-date information on *A. torrentium* in Italy. It was carried out to fill the knowledge gap on the distribution of this species in Italy, after the Italian Ministry of Environment, Land and Sea Conservation described the information on *A. torrentium* as "data deficient" during the preparations for the Habitats Directive reports to the EU Commission for the period 2013–2018 (Prot. 0017966 PNM of 17/09/2015) and the occurrence of the species was classified as uncertain or with Scientific Reserve (SCR).

Of the stations where eDNA screening yielded a positive result for the presence of *A. torrentium*, three (1, 2, and 3) were known locations for *A. torrentium* [19], while Stations 5, 10, and 12 represented new findings. Station 1 is a known and the most northern location of *A. torrentium* in Italy; Stations 2, 3, 10 and 12 are located in a cluster of several kilometres to the south-east along the Slizza and are all grouped around Rutte Piccolo sampling sites (Stations 2 and 3). Station 5 is located several kilometres eastwards and is near a site where *A. torrentium* occurred in the past (in the 1990s') but is now likely extinct (stream near the border with Slovenia [55,56]). The absence of the crayfish was also observed in this study by monitoring campaigns conducted for two consecutive years and by a negative eDNA signal

in our study (Station 4). However, since *A. torrentium* can actively migrate over shorter distances [57], the crayfish at these two sites could belong to the same metapopulation. In addition to Station 4, we also obtained a negative signal in eDNA tests from two sites near historically known populations of *A. torrentium* (Stations 7 and 8), also indicating possible local extinctions. Station 9 is the only sampling location that provided an uncertain result—the observation of a signal in only one technical replicate. Such an observation could be an artefact, but it could also indicate very low abundance, especially since the same pattern was observed in two out of three independent samplings.

This eDNA survey, along with field observations [17,19], shows that the stone crayfish still survives in four small streams in the River Slizza watershed (Stations 1–3, 5, 10 and 12). However, a negative trend in the population status has been observed and indicates possible local extinctions in the Italian part of the Slizza watershed. Therefore, conservation actions and management plans for this species are urgently needed. We suggest that future efforts should focus on field monitoring and on eDNA surveys of stations where local extinction is suspected (Stations 4, 7 and 8), on Station 9 where eDNA tests were uncertain, and on searching for this species in new locations in the Slizza watershed.

Fall sampling at a subset of the stations revealed that although eDNA detectability depends on the sampling season, eDNA detection of crayfish is possible also in the fall. The lower success of detection in fall is most evident from the Ct values at which the signal was observed. In fall, after correction for inhibition, their average was 35.6, while in summer, it was 33. The lower temperatures in fall result in lower activity and metabolism, probably leading to a lower eDNA shedding rate. However, mating and spawning also occur in late October [7]. Seasonality in the detection of eDNA due to differences in animal activity has been observed in many studies [58–60] and has also been observed in *A. torrentium* [33] and other crayfish species [61,62]. Although some studies suggest that peak eDNA levels are linked to reproduction [26,63], the seasonal dynamics of eDNA in relation to population size are poorly understood. However, a recent study on *A. pallipes* showed that the detection probabilities of technical replicates vary considerably, ranging from 20% to over 80% between seasons [64]. Furthermore, different life stages of semiaquatic species may influence the seasonally varying eDNA contribution [65].

Species-specific qPCR assays for detecting eDNA from water samples are a valuable technique for monitoring endangered, rare, invasive and cryptic species [66], and the set of primers presented here succeeded in distinguishing between *A. torrentium* and *A. pallipes*. The *Ator* assay detected a positive signal for *A. torrentium* in eDNA samples collected at six Stations (1–3, 5, 10 and 11) in the Slizza watershed. In contrast, the *Apal* assay detected a positive signal for *A. pallipes* only at one Station 15 in the Tagliamento basin. The absence of *A. pallipes* in the eDNA samples from the Slizza basin confirms that *A. pallipes* never colonised or was transferred into this area, also confirming conclusions of Machino [6] and Machino and Füreder [17] that historical observations of *A. pallipes* here are identification mistakes.

The common reverse primer developed in our study, together with specific forward primer and probe oligonucleotides, allowed us to target the same region of 16S rRNA while discriminating between *A. torrentium* and *A. pallipes*. Both assays showed high specificity and no cross-amplification between the two target species and with non-native decapods (*P. clarkii* and *F. limosus*) that (potentially) occur in the region [44–46].

In silico analysis showed that the *Apal* assay should amplify all haplotypes of *A. pallipes* known to occur in the FVG. Sixteen haplotypes fully matched our assay, while one, haplotype 6 (hap6_37ROS; GenBank OM422812), had a single mismatch within the forward primer. Sequences with two or more mismatches are predominantly found in Spain, Germany, France and Italy (Po tributary) (GenBanks Accession Numbers are listed in File S1B). However, none of the mismatches are located near the 3′ end of the primer, which increases the probability of a successful detection.

In silico analysis also revealed high specificity for the *Ator* assay. The network analysis approach included haplotypes from all *A. torrentium* pyhlogroups as defined

by Lovrenčić et al. [54] (Central and SE Europe—CSE, Southern Balkans—SB, Lika and Dalmacitia—LD, Banija—BAN, Kordun—KOR, Zelen Vir—ZV, Gorski Kotar—GK, Žumberak, Plitvice and Bjelolasica—ŽPB), except those from Apuseni Mountains—APU. Most of the haplotypes completely matched the assay, while a few of them contained a single mismatch in the forward primer. The haplotypes with a mismatch were from Croatia and Bulgaria [67], which is not surprising since Croatia and north-central Dinarides (Bosnia and Herzegovina) generally have the highest genetic diversity of *A. torrentium* in all of Europe [54]. The network analysis approach used here is advantageous to evaluate the usability of a qPCR assay for eDNA detection of genetically highly heterogenous species distributed over a large geographical area. Overall, the in silico analysis demonstrated that the two assays are likely to be useful for monitoring the distribution of *A. torrentium* and *A. pallipes* outside the study area with the eDNA approach. The assays should detect 16S rRNA from the most known phylogroups of these two species, including the Western Balkan populations of *A. pallipes*, where multiple phylogroups coexist in a relatively narrow geographical area [67,68]. Nevertheless, caution should also be exercised when using *Ator* and *Apal* assays in other geographical areas, and in vitro tests should first be performed on gDNA from local samples.

5. Conclusions

Increasing monitoring of both species allows researchers and stakeholders to quickly collect data on target species faster than traditional field monitoring, which often requires multiple visits to the same site. This approach is also a perfect candidate for preliminary monitoring over broad areas in advance of more focused efforts (on sites with positive detections). In this sense, the potential of eDNA application could also lead to future low-cost monitoring plans in the preliminary phase and then allocation of a larger budget to monitoring sites that tested positive/uncertain in the preliminary phase. Furthermore, it is possible to detect and analyse more than one species from the same filtered sample, as in this case, and thus each extracted eDNA is representative of the predominant species inhabiting the same site. Another important consideration is that, if properly stored, the eDNA could be further analyzed to screen for additional species that are not targeted for monitoring at the outset of a management plan without the need for a second field sampling.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/d14030205/s1. File S1A: List of the GenBank IDs of *A. torrentium* sequences used to design and test *Ator* primers; File S1B: List of the GenBank IDs of *A. pallipes* sequences used to design and test *Apal* primers; File S1C: List of the GenBank IDs of invasive crayfish species used to test species-specificity of both *Ator* and *Apal* assays; Figure S2: Haplotype network of *A. pallipes* 16S rDNA of sequences collected locally in the Friuli Venezia Giulia (FVG); File S3: Full list of the sequences used in the *Ator* network; File S4: Full list of the sequences used in the *Apal* network; Figure S5: Haplotype network of the *A. pallipes* 16S sequences together with the Ator assay; Figure S6: Haplotype network of the *A. torrentium* sequences together with the Apal assay; File S7.1: Limit of detections (LODs) calculation and data analysis; Figure S7.2: Standard curve plot of the qPCR positive control samples; File S8.1: Inhibition test; Figure S8.2: Detail of the Standard curve for Internal Amplification Control (IAC); File S9: Detailed results obtained for each sampled site reported in Figures 4 and 5.

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