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Early Molecular Detection of Invasive Alien Plants in Urban and Peri-Urban Areas

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Abstract: Invasive alien plants represent one of the five major threats to biodiversity and the disruption of ecosystems. They are introduced through various routes, starting with commercial trade. Preventing their introduction is essential to avoid the spread of new invasive plants. In this paper, we propose a new early warning DNA barcoding tool for invasive plant detection. Eight invasive alien species of European Union concern (i.e., *Ludwigia grandiflora*, *Elodea nuttallii*, *Myriophyllum aquaticum*, *Pontederia crassipes*, *Ailanthus altissima*, *Heracleum mantegazzianum*, *Impatiens glandulifera*, *Pueraria montana*) were selected and analysed. A unique DNA marker for each species was identified and amplified using species-specific primers capable of identifying the presence of alien species. To verify whether the approach could detect the presence of alien plants in urban areas from lawn clippings, mixes with typical urban spontaneous plants and invasive species were tested. In all mixes, only the invasive species was identified. This rapid detection capability will enable environmental operators to intervene promptly to contain the spread of invasive plants before they can cause significant damage to the local ecosystem. This tool could have a significant impact on the protection of local biodiversity and the integrity of urban habitats.

Keywords: invasive alien species; invasive alien plant species; DNA barcoding; urban area; biodiversity; biodiversity loss

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1. Introduction

Invasive alien species (IAS) have been defined by the latest United Nation's (UN) Intergovernmental Platform for Biodiversity and Ecosystem Services (IPBES) report as those whose presence in a specific region is due to human activities [1] and that, through their spread, show a negative impact on biodiversity, local ecosystems and other species [2]. The European Commission has defined alien species as those that, upon introduction or spread, have been found to threaten or adversely impact biodiversity and related ecosystem services [3]. Globally, plants represent the second IAS in terms of abundance (i.e., 1061), just below invertebrates (i.e., 1852) and followed by vertebrates (i.e., 461) and microorganisms (i.e., 141) [4]. These figures, which are probably underestimated, will likely increase due to global change in the near future, thereby enhancing the extent and impact of invaders on the ecosystem [5]. Invasive alien plant species (IAPS) represent serious threats to local biodiversity, ecosystem services, environmental quality and also cause health problems [6]. IAPS are known to negatively affect ecosystem functioning and structure mainly through different actions, including the following: (i) decreasing overall diversity in terms of native (i.e., animals and plants) species; (ii) altering soil (physical and chemical) properties and microbial community functioning; (iii) changing firewood regime (i.e., frequency and

intensity) [7]. IAPS are also responsible for impacting ecosystem services, including a decrease in several provisioning [8] and regulatory services [9]. IAPS represent a serious concern mainly in high-income countries, where very active human-mediated activities (transport, migration and commerce) are responsible for higher (30-fold) numbers of alien species in comparison to those in developing countries [10].

In Europe, the financial impact of invasive alien species from 1960 to 2020 was recently estimated at EUR 116.61 billion [11]. However, due to ongoing data gaps, this figure is likely underestimated [12]. It should be noted that the costs associated with invasive alien species (IAS) are not limited to directly quantifiable damages or management expenses. They also encompass various indirect costs that are difficult to quantify, such as ecosystem services [13,14].

Urban areas are known to be hotspots for IAPS diffusion because of their pronounced habitat heterogeneity [15,16], higher average air temperatures due to the heat island phenomenon (which enables the spread of thermophilic species) and the elevated hazard of IASP propagules from ornamental horticulture (public and private gardens) [17]. Urban regeneration processes and urban forestry activities are increasing green spaces in cities and enhancing ecological connections with urban parks and protected areas. While this undoubtedly has benefits for nature, it also raises the risk of spreading invasive alien species. Monitoring the presence of IAPS is fundamental to prevent their spread in the environment, especially in the young growth phase, small seedlings and/or seeds and propagation material [18]. Traditionally, invasive species detection and monitoring have heavily relied on morphological features [19,20]. However, considering that it is difficult to morphologically analyse plants in the early stages of development, it is essential to have early detection systems based on other markers, such as DNA. DNA barcoding has been increasingly used for the identification of invasive species [21]. This is a molecular technique that uses a short genetic sequence from a standardised region of the genome to identify species. For plants, common barcoding regions include the ribulose-bisphosphate carboxylase (*rbcL*) and maturase K (*matK*) genes [22,23]. However, even though these regions are commonly used for DNA barcoding in plants due to their moderate variability and ease of amplification, they often lack sufficient variability to distinguish closely related species effectively. To address this issue, the *psbA-trnH* intergenic spacer and the nuclear ribosomal internal transcribed spacer 2 (ITS2) region exhibit higher variability and are more suitable for plant species identification [24]. These markers provide better resolution at the species level and have shown higher success rates in invasive species identification [20]. Although this approach is scientifically rigorous, it needs to be easily applicable by regional agencies responsible for environmental and biodiversity monitoring. For this reason, in this work we aimed to evaluate the following: (i) the effectiveness of developing species-specific primers capable of amplifying DNA barcode regions of only alien species; (ii) the specificity of these markers against commonly distributed urban herbaceous species in Europe; (iii) an efficient analytical procedure to propose to regional agencies. The result of this study could represent a significant step forward in the management of invasive species in urban environments, contributing to the preservation of biodiversity and the protection of urban ecosystems.

2. Materials and Methods

2.1. Invasive Alien Species of European Union Concern: Selection and Collection

All invasive plant samples belonging to the list of invasive alien species of Union concern (https://environment.ec.europa.eu/topics/nature-and-biodiversity/invasive-alien-species_en accessed on 1 October 2024) were obtained from Trauttmansdorff, Bozen (Italy), where in 2023, the exhibition "Invasive Neophytes" (www.trauttmansdorff.it/en/Magazin/Invasive-Neophytes accessed on 1 October 2024) aimed to share information and concerns about these invasive plant species. Samples were carefully harvested under the strict supervision of the Trauttmansdorff personnel and processed on site to avoid the risk of spreading propagules across the environment. At sampling, only eight species were available for

assessment (Table 1), with four belonging to aquatic environments (i.e., *Elodea nuttallii* (Planch.) H.St.John, *Ludwigia grandiflora* (Michx.) Greuter & Burdet, *Myriophyllum aquaticum* (Vell.) Verdc. and *Pontederia crassipes* (Mart.)) and four to terrestrial environments (i.e., *Ailanthus altissima* (Mill.) Swingle, *Heracleum mantegazzianum* Sommier & Levier, *Impatiens glandulifera* Royle and *Pueraria montana* (Lour.) Merr.).

Table 1. List of invasive species of EU concern used in this trial collected in Trauttmansdorff, Bozen (Italy).

Sample ID	Botanical Name	Family	Origin	Habitus
SI_IG01	*Impatiens glandulifera* Royle	Balsaminaceae	Asia	Annual herbaceous
SI_HM02	*Heracleum mantegazzianum* Sommier & Levier	Apiaceae	Caucasus	Perennial herbaceous
SI_PM03	*Pueraria montana* (Lour.) Merr.	Fabaceae	East Asia	Herbaceous perennial vine
SI_AA04	*Ailanthus altissima* (Mill.) Swingle	SimaroubaceaeAmaranthaceae	Northern and central China	Shrub/Tree
SI_EN05	*Elodea nuttallii* (Planch.) H.St.John	Hydrocharitaceae	North America	Perennial aquatic
SI_LG06	*Ludwigia grandiflora* (Michx.) Greuter & Burdet	Onagraceae	South America	Perennial aquatic
SI_PC07	*Pontederia crassipes* (Mart.)	Pontederiaceae	South America	Perennial aquatic
SI_MA08	*Myriophyllum aquaticum* (Vell.) Verdc.	Haloragaceae	South America	Perennial aquatic

2.2. Spontaneous Plant Collection

To simulate lawn clippings for creating mixes in the laboratory, two actual field sites in the Milan metropolitan area (Italy) were sampled for plant assessment in both terrestrial and aquatic environments (Figure 1). For the terrestrial environment, a site near Milan airport in Malpensa was chosen because this type of site, where both native and alien plants co-occur, may represent a hotspot for the introduction of alien species in the region [25]. In particular, the site is an open abandoned field (0.5 ha) on the outer edge of the eastern border of the airport, where a spontaneous mix of tree, shrub and herbaceous species coexist. The aquatic site (1 ha) was located within a natural park (Parco del Ticino) and constituted of a network of canals used for irrigation. One plant survey for each site was performed in late summer 2023. For both sites, five (4 × 4 m) square relevé plots were selected, representing the average conditions of the site in terms of vegetation uniformity (i.e., composition and structure) as well as habitat type. In both cases, the plant survey was performed using a systematic approach. The sampling of the vegetation was performed only by assessing the presence/absence of a new plant species without counting their number. Plant identification was performed using available keys. The botanical nomenclature followed the "Portal to the Flora of Italy" (http://dryades.units.it/floritaly accessed on 1 October 2024), and for alien species, the "Plants of the World Online" database (https://powo.science.kew.org, accessed on 1 October 2024). The species collected are shown in Table 2.

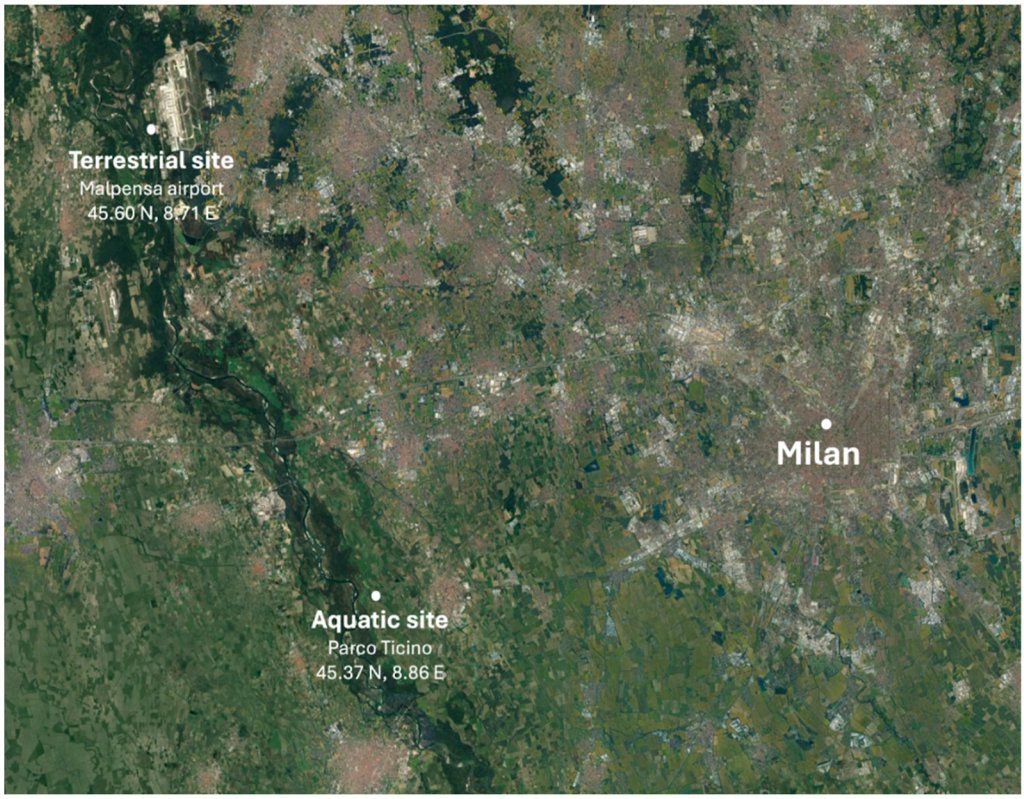

Figure 1. Location of the sampling sites.

Table 2. In the table are indicated the mixes prepared in the laboratory, containing 25 mg of each plant species (both invasive and spontaneous vegetation species).

Aquatic				Terrestrial			
MIX 1	**MIX 2**	**MIX 3**	**MIX 4**	**MIX 5**	**MIX 6**	**MIX 7**	**MIX 8**
Ludwigia grandiflora (Michx.) Greuter & Burdet	*Elodea nuttallii* (Planch.) H.St.John	*Myriophyllum aquaticum* (Vell.) Verdc.	*Pontederia crassipes* (Mart.)	*Ailanthus altissima* (Mill.) Swingle	*Heracleum mantegazzianum* Sommier & Levier	*Impatiens glandulifera* Royle	*Pueraria montana* (Lour.) Merr.
Phragmites australis (Cav.) Trin. ex Steud. *Typha latifolia* L. *Helosciadium nodiflorum* L. W.D.J.Koch *Callitriche stagnalis* (Scop.) *Myriophyllum spicatum* L. *Groenlandia densa* (L.) Fourr *Stuckenia pectinata* (L.) Börner *Elodea canadensis* Michx.				*Acer negundo* L. *Achillea nobilis* L. *Artemisia verlotiorum* Lamotte *Buddleja davidii* Franch. *Chelidonium majus* L. *Erigeron annuus* (L.) Desf. *Erigeron canadensis* L. *Galinsoga quadriradiata* Ruiz & Pav. *Geranium molle* L. *Juncus effusus* L. *Lolium perenne* L. *Malva sylvestris* L. *Oxalis dillenii* Jacq. *Phytolacca americana* L. *Plantago lanceolata* L. *Plantago major* L. *Rumex acetosella* L. *Rumex pulcher* L. *Setaria pumila* (Poir.) Roem. & Schult. *Silene vulgaris* (Moench) Garcke *Solidago gigantea* Aiton *Taraxacum officinale* F.H.Wigg. *Trifolium pratense* L. *Trifolium repens* L.		

2.3. Primer Pair Design

Primer pairs for species-specific identification were newly designed in silico. All nucleotide sequences of the Internal Transcribed Spacer 2 (ITS2) and *psbA-trnH* region for the invasive species genera in Table 1 were obtained from NCBI Nucleotide and were aligned using the latest version of ClustalOmega software (www.ebi.ac.uk/Tools/msa/clustalw2/ accessed on 1 October 2024). The most variable regions for the target species were identified using Bioedit software (7.7.1. version), and a primer pair specific to all the species in the study was designed de novo. All the primer couples were tested for species specificity using the Primer–Blast tool available from NCBI (www.ncbi.nlm.nih.gov/tools/primer-blast/ accessed on 1 October 2024) to verify the specificity for the target species. The primer pairs were ordered from an external service (Eurofins, Hamburg, Germany).

2.4. DNA Barcoding Analysis

The total genomic DNA was extracted from 80 mg ± 10 of plant species samples using the Qiagen© DNeasy® Plant Pro Kit (Qiagen, Hilden, Germany), with an added thermal lysis step (65 °C for 1 h). DNA quantification was performed using the Qubit™ dsDNA HS Assay Kit (Thermofisher, Waltham, MA, USA). Firstly, all eight plants were tested for species authenticity by performing DNA barcoding analysis. Then, 25 µL PCR amplification using illustra™ PuReTaq RTG PCR Beads (Merck, Darmstadt, Germany) was performed following the ensuing programme: 95 °C for 5 min, 35 cycles of 95 °C for 45 s, 55 °C for 45 s, 72 °C for 1 min and finally 10 min at 72 °C. The Internal Transcribed 2 (ITS2) marker was chosen as the marker for the identification for its high sequencing success rate [26], as well as its short length, ease of amplification and high discriminatory power [27]. Amplicon occurrence was assessed by capillary electrophoresis (QIAxel connect, Qiagen, Germany). Amplicon length was measured by comparison against the QX Alignment Marker 15 bp/600 bp (Qiagen, Germany). Purified PCR products were sent to an external service (Macrogen, Amsterdam, The Netherlands) for sequencing and edited as described in Frigerio et al. [28]. The obtained sequences were analysed after using the NCBI Nucleotide Blast tool (https://blast.ncbi.nlm.nih.gov/Blast.cgi accessed on 1 October 2024). Each barcode sequence was taxonomically assigned to the plant species with the nearest matches (maximum identity >99% and query coverage of 100%). Then, another PCR amplification was performed on invasive species to test the primer pairs designed following the same protocol described above, changing the annealing temperature from 55 °C to 59 °C.

2.5. Creation of Mixes and Testing of Primer Pairs

To test the ability of our method to detect the presence of invasive species within lawn clippings samples, eight mixes were created in the laboratory and tested. Each mix contained 25 mg of each of the 8 invasive species (i.e., dry weight) and 25 mg of each 8 to 24 (i.e., dry weight, respectively, for the aquatic and terrestrial mixes) spontaneous vegetation species (Table 2). The DNA from the mixes was extracted following the same protocol described in Section 2.3, tested with the species-specific primers designed, and the obtained amplicons were purified, sequenced by Macrogen (Europe) and analysed using the NCBI Nucleotide Blast tool (https://blast.ncbi.nlm.nih.gov/Blast.cgi accessed on 1 October 2024). Each barcode sequence was taxonomically assigned to the plant species with the nearest matches (maximum identity >99% and query coverage of 100%).

2.6. Limit of Detection and Multiplex PCR

To evaluate the limit of detection (LOD) of the test, the eight mixes' DNA extracts were diluted from 10^{-1} up to 10^{-5} and tested with PCR. Thereafter, a multiplex PCR was performed by combining two of the invasive species with different amplicon lengths. Precisely we combined *Pontederia crassipes* (i.e., 350 bp) and *Myriophyllum aquaticum* (i.e., 249 bp), *Impatiens glandulifera* (i.e., 273 bp) and *Ludwigia grandiflora* (i.e., 494 bp), *Heracleum mantegazzianum* (i.e., 225 bp) and *Pueriaria montana* (i.e., 322 bp) and finally *Ailanthus altissima* (i.e., 218 bp) and *Elodea nuttallii* (i.e., 320 bp). Both the limit of detection (LOD) and

multiplex PCR were conducted following the PCR amplification programme described in Section 2.3.

3. Results

3.1. DNA Barcoding

DNA extraction was successful for all eight invasive species and their mixes, with a good quantity of DNA (60 ng/µL ± 20 ng/µL). The DNA barcoding analysis for invasive species identification was performed, confirming the declared species, as shown in Table A1.

3.2. Primer Pair Design and Test on Invasive Species

The species-specific primer pairs obtained, listed in Table 3, were designed by evaluating the CG content (~45%), the difference in annealing temperature between the forward and reverse primers (~1 °C), and the amplicon length (200–500 bp), and identifying the most variable region.

Table 3. Species-specific primer pairs designed: the table shows the primer region marker used for the in silico design, the primer specificity for the invasive species, the amplicon length, the annealing temperature and the 5′-3′ sequence of both the forward and reverse primers.

Primer Name	Primer Region	Primer Specificity	Amplicon Length	Annealing Temperature	Sequence (5′-3′)
SI_IG01_F SI_IG01_R	ITS2	*Impatiens glandulifera*	273 bp	60 °C	TATGGAGCAGTTGGCCGAAA AACGACGAAGCCGTTCGATT
SI_HM02_F SI_HM02_R	ITS2	*Heracleum mantegazzianum*	225 bp	60 °C	CCCACAACCACACACTCCTT CTGGGGTCACAGTCGAAGC
SI_PM03_F SI_PM03_R	*psbA-trnH*	*Pueraria montana var. lobata*	322 bp	59 °C	TCCGTCCATCAAAATTCCAGG GAGGGACTTGAACCCTCACG
SI_AA04_F SI_AA04_R	ITS2	*Ailanthus altissima*	218 bp	60 °C	ATCGGTGGCGGAAATTCCAT CGATTCTCAAGCTGGGCTCT
SI_EN05_F SI_EN05_F	ITS2	*Elodea nuttallii*	320 bp	60 °C	TGCCTGGGAGTCTTTTCGAC TAAACTCAGCGGGTGACCAT
SI_LG06_F SI_LG06_R	*psbA-trnH*	*Ludwigia grandiflora*	494 bp	59 °C	CATCCGCCCCTTAACTCTCAT AAGACTTCCGTCTTAGTGTAAGTG
SI_PC07_F SI_PC07_R	ITS2	*Pontederia crassipes*	325 bp	59 °C	ACGGATTGTTGAGGTAAATTGGC GGAGTAATCCACTGTGACACG
SI_MA08_F SI_MA08_R	ITS2	*Myriophyllum aquaticum*	249 bp	60 °C	TCGCGAGAAGTCCACTGAA GTTATTGTAGCCGAGGGCGA

As shown in Figure 2, all the invasive species were successfully amplified and the amplicon length matched with the values reported in Table 3. Therefore, for all the specimens it was possible to identify the species, proving the ability of these primer couples to correctly amplify and identify all invasive species that were the subject of this study. The sequences were deposited on GenBank, and the accession numbers are shown in Table A1.

3.3. Species-Specific Primer Testing on Mixes and Limit of Detection

The mixes' DNA extracts were successfully amplified by testing the species-specific primer pairs, and the sequences obtained matched with the invasive species, confirming the ability of the primer pairs to detect the presence of invasive species within the mixes (Table A2; Figure 3).

For the limit of detection, three microlitres of mix DNA dilutions from 10^{-1} up to 10^{-5} were tested. For mixes 2 and 3, the species were detected up to a 10^{-5} dilution; in mixes 6 and 8, up to 10^{-4}; in mixes 4, 5 and 7, up to 10^{-3}; and finally, in mix 1, up to 10^{-2}. The results of the PCR reaction showed the high sensitivity of this assay.

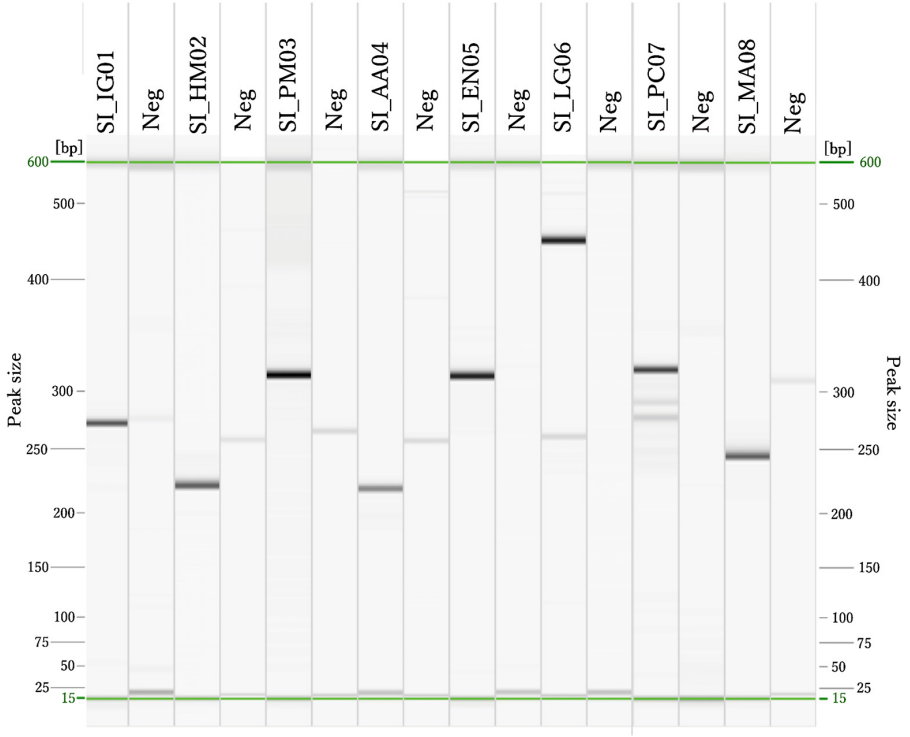

Figure 2. The picture shows the PCR amplification of each invasive species (Table 1) using the primer pairs designed in this study (Table 3) and the negative samples.

3.4. Multiplex PCR

From the perspective of saving time and resources, a multiplex PCR was performed. Each pair of invasive species was combined and amplified, but the first two combinations, containing *Pontederia crassipes* with *Myriophyllum aquaticum* (i.e., SI_PC07 + SI_MA08) and *Impatiens glandulifera* with *Ludwigia grandiflora* (i.e., SI_IG01 + SI_LG06), resulted in the amplification of only one species (i.e., *Pontederia crassipes* and *Impatiens glandulifera*) (Figure 4). Otherwise, the other two combinations, containing *Heracleum mantegazzianum* with *Pueraria montana* (i.e., SI_HM02 + SI_PM03) and *Ailanthus altissima* with *Elodea nuttallii* (i.e., SI_AA04 + SI_EN05), were successfully amplified and separated (Figure 4).

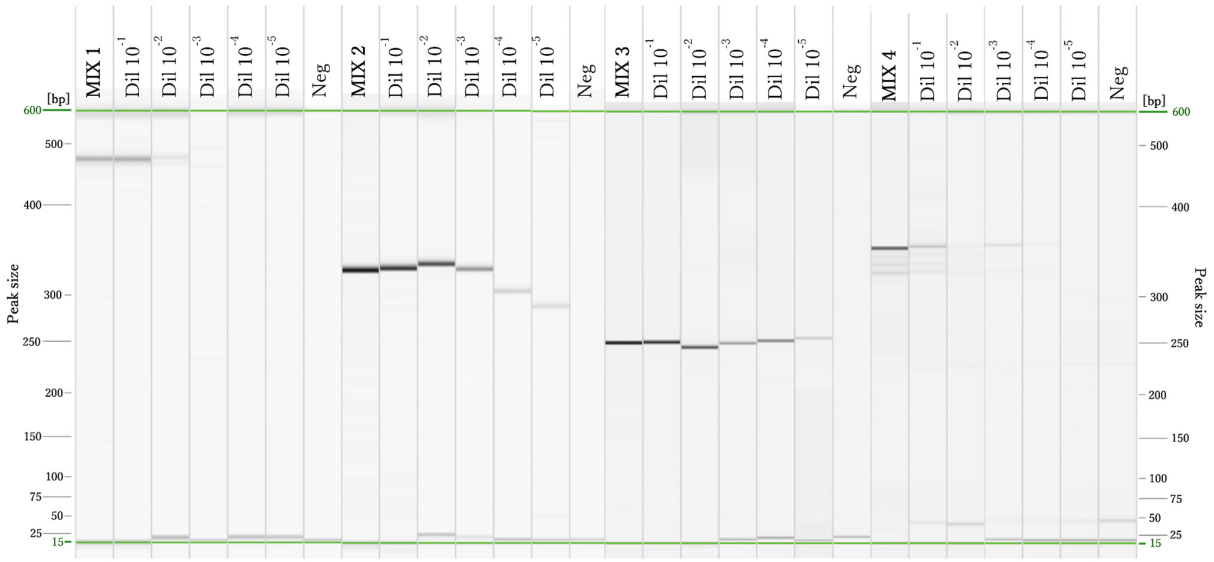

Figure 3. *Cont.*

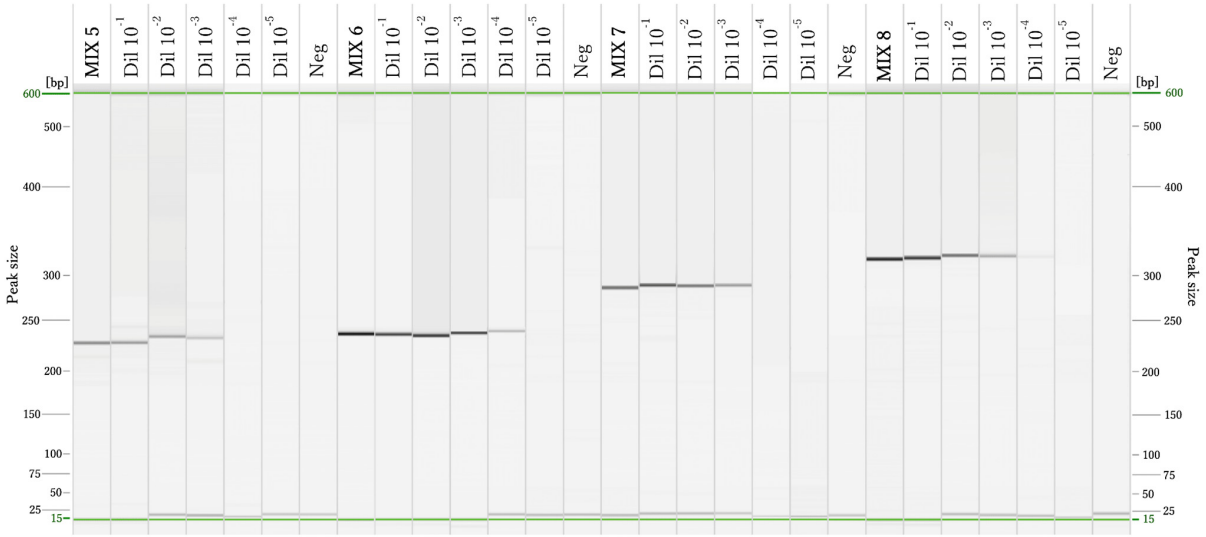

Figure 3. PCR amplification of the mixes' extracts (Table 2) and their dilution from 10^{-1} up to 10^{-5}.

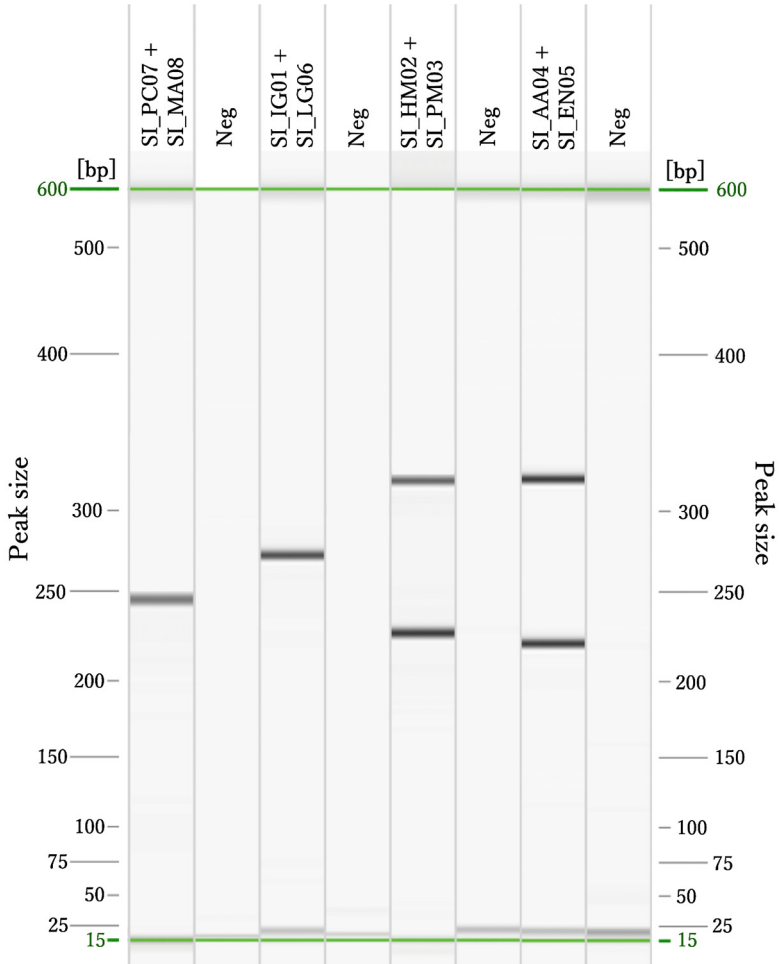

Figure 4. Multiplex PCR of the invasive species combinations. As shown in the picture, in the first two combinations, the PCR only amplified one species (i.e., *Pontederia crassipes* and *Impatiens glandulifera*), while the other two combinations were successfully separated (i.e., *Heracleum mantegazzianum* with *Pueraria montana* and *Ailanthus altissima* with *Elodea nuttallii*).

4. Discussion

In this research, a quick and efficient method for the early detection of invasive species using the power of DNA barcoding and species-specific primers was developed. This tool efficiently identifies the invasive species even in the mixes created in the laboratory, which simulate samples of lawn clippings from urban areas where invasive species can make their way into parks, reserves and peri-urban areas. Indeed, it is essential to simulate what could be a routine inspection for municipality monitoring and, at the same time, provide environmental agency operators with simple tools to detect these species.

Identifying invasive species at the initial stages of their introduction allows for prompt intervention, which is essential for preventing their establishment and subsequent spread [29]. When invasive species are detected early, management actions such as eradication, containment and control can be implemented before the species becomes widespread [30,31]. This not only minimises the ecological disruption caused by the invasive species but also reduces the costs associated with long-term management efforts. In this context, the simplicity and sensitivity of the method are crucial. Our approach allowed us to develop a highly sensitive method, as it detected the presence of invasive species from a very small amount of DNA, which could come from a single small leaf or plant fragment.

In urban environments, where human activities and trade can introduce invasive species more frequently [32], early detection systems are especially important. Implementing an efficient early warning tool based on DNA barcoding can significantly enhance the ability to monitor and respond to invasive species threats. By identifying invasive species before they become problematic, urban areas can safeguard their green spaces, protect native biodiversity and avoid the extensive costs associated with late-stage invasive species management [29]. In this context, the speed of the analysis becomes crucial. The analysis developed in this study provides results within a few hours. Additionally, due to the reduction in sequencing costs in the last few years, it is also inexpensive. The financial impact of invasive alien species (IAS) can cause extensive economic damage [33], but our method could reduce eradication costs by preventing the spread of invasive species. It is also worth noting that this method is very cost-effective, and it is possible to extract and amplify DNA within a few hours for less than 10 euros.

Although the technique developed in this study has the advantage of offering high specificity and adaptability, allowing for rapid, on-site identification without requiring extensive taxonomic knowledge or specialised laboratory equipment, it does have some limitations. Its main disadvantage lies in the fact that it is a target analysis, so can only detect pre-selected species, making it less effective when multiple or unknown invasive species are present. However, other methodologies known in the literature can compensate for these shortcomings. eDNA metabarcoding, for example, allows for the simultaneous identification of multiple species within an environmental sample, making it ideal for broad biodiversity assessments. This technique is highly efficient in detecting diverse communities, particularly in complex ecosystems. However, its disadvantages include the need for advanced laboratory facilities and a reduced ability to differentiate closely related species. Additionally, the method requires complex data analysis and interpretation compared to species-specific DNA barcoding, and it often involves longer processing times to obtain results [34]. Both techniques have their strengths: species-specific DNA barcoding is better suited for targeted analysis and quick detection, while metabarcoding is more appropriate for large-scale ecological surveys. For the purposes of this study, which required specific, fast and cheap species detection, the use of species-specific primers based on DNA barcoding represented the optimal solution.

To turn the tool developed in this study into one for routine analysis without specialised technical personnel and a well-equipped laboratory, future studies could be based on applying this technique and the primer pairs developed in this study to rapid technologies such as LAMP and RPA. These are emerging techniques with recent applications in biosecurity and forensic sciences [35–37] and that can be used as field-based detection tools. These techniques allow for the development of a targeted method for species identi-

fication through a colorimetric or visual test. In the literature, they are typically used for bacterial detection and food traceability [38–40]. However, it is possible to adapt them to any target, including invasive species of interest. Although laboratory verification through Sanger sequencing is recommended to confirm the species, it can still be a helpful tool for municipalities to take quick action and contain the spread of invasive species.

5. Conclusions

This study demonstrates that by utilising DNA barcoding analysis, it is possible to develop species-specific primers for invasive species. It highlights that this approach is (i) adaptable to different environmental contexts and target species; (ii) sensitive and does not require extensive taxonomic knowledge; (iii) represents a system that can be adopted by various environmental monitoring actors, providing them with an additional tool in the fight against invasive species. This method could also be used at border offices, customs, airports and seaports to perform checks on goods that might introduce invasive exotic species. In particular, to allow for rapid and on-site identification without the need for specialised laboratories, the method developed in this study can be applied to rapid techniques with visual detection by the naked eye.

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Conflicts of Interest: The authors declare no conflicts of interest.

Appendix A

Table A1. The table shows the results of DNA barcoding identification of invasive plant species, confirming their authenticity.

Sample Code	Marker	Declared Species	Obtained Species	Sample DNA Concentration (ng/μL)	Accession Number
SI_IG01	ITS2	*Impatiens glandulifera*	*Impatiens glandulifera*	29.7	PQ435191
SI_HM02	ITS2	*Heracleum mantegazzianum*	*Heracleum mantegazzianum*	45.1	PQ435192
SI_PM03	ITS2	*Pueraria montana*	*Pueraria montana*	28.7	IASIT001-24
SI_AA04	ITS2	*Ailanthus altissima*	*Ailanthus altissima*	60.35	PQ435193
SI_EN05	ITS2	*Elodea nuttallii*	*Elodea nuttallii*	60.5	PQ435194
SI_LG06	ITS2	*Ludwigia grandiflora*	*Ludwigia grandiflora*	15.6	IASIT002-24
SI_PC07	ITS2	*Pontederia crassipes*	*Pontederia crassipes*	17.2	IASIT003-24
SI_MA08	ITS2	*Myriophyllum aquaticum*	*Myriophyllum aquaticum*	31.8	PQ435195

Table A2. The table shows the results of DNA barcoding identification of mixes, confirming the ability of primer pairs to identify the invasive species. The sequences were submitted to public databases.

Mix Code	Species Contained	Obtained Species	Sample DNA Concentration (ng/µL)
MIX 1	*Ludwigia grandiflora*	*Ludwigia grandiflora*	10.1
MIX 2	*Elodea nuttallii*	*Elodea nuttallii*	5.86
MIX 3	*Myriophyllum aquaticum*	*Myriophyllum aquaticum*	15.5
MIX 4	*Pontederia crassipes*	*Pontederia crassipes*	10.2
MIX 5	*Ailanthus altissima*	*Ailanthus altissima*	6.8
MIX 6	*Heracleum mantegazzianum*	*Heracleum mantegazzianum*	5.9
MIX 7	*Impatiens glandulifera*	*Impatiens glandulifera*	34.8
MIX 8	*Pueraria montana*	*Pueraria montana*	2.32

References

1. de Barros Ruas, R.; Costa, L.M.S.; Bered, F. Urbanization driving changes in plant species and communities—A global view. *Glob. Ecol. Conserv.* **2022**, *38*, e02243. [CrossRef]
2. Roy, H.E.; Pauchard, A.; Stoett, P.; Renard Truong, T.; Bacher, S.; Galil, B.S.; Hulme, P.E.; Ikeda, T.; Sankaran, K.; McGeoch, M.A.; et al. *IPBES Invasive Alien Species Assessment: Summary for Policymakers (Version 3) Zenodo*; University of Rhode Island: South Kingstown, RI, USA, 2024. [CrossRef]
3. European Union. Regulation (EU) No 1143/2014 of the European Parliament and of the Council of 22 October 2014 on the prevention and management of the introduction and spread of invasive alien species. *Off. J.* **2014**, *317*, 35–55.
4. IPBES. *Global Assessment Report on Biodiversity and Ecosystem Services of the Intergovernmental Science-Policy Platform on Biodiversity and Ecosystem Services*; Brondizio, E.S., Settele, J., Díaz, S., Ngo, H.T., Eds.; IPBES Secretariat: Bonn, Germany, 2019; 1148p. [CrossRef]
5. Pyšek, P.; Hulme, P.E.; Simberloff, D.; Bacher, S.; Blackburn, T.M.; Carlton, J.T.; Dawson, W.; Essl, F.; Foxcroft, L.C.; Genovesi, P.; et al. Scientists' warning on invasive alien species. *Biol. Rev.* **2020**, *95*, 1511–1534. [CrossRef]
6. Rai, K.P.; Singh, J.S. Invasive alien plant species: Their impact on environment, ecosystem services and human health. *Ecol. Indic.* **2020**, *111*, 106020. [CrossRef]
7. Pyšek, P.; Jarošík, V.; Hulme, P.E.; Pergl, J.; Hejda, M.; Schaffner, U.; Vilà, M. A global assessment of invasive plant impacts on resident species, communities and ecosystems: The interaction of impact measures, invading species' traits and environment. *Glob. Chang. Biol.* **2012**, *18*, 1725–1737. [CrossRef]
8. Pejchar, L.; Mooney, H.A. Invasive species, ecosystem services and human well-being. *Trends Ecol. Evol.* **2009**, *24*, 497–504. [CrossRef]
9. Colautti, R.I.; Bailey, S.A.; Van Overdijk, C.D.; Amundsen, K.; MacIsaac, H.J. Characterised and projected costs of nonindigenous species in Canada. *Biol. Invasions* **2006**, *8*, 45–59. [CrossRef]
10. Seebens, H.; Blackburn, T.M.; Dyer, E.E.; Genovesi, P.; Hulme, P.E.; Jeschke, J.M.; Pagad, S.; Pyšek, P.; van Kleunen, M.; Winter, M.; et al. Global rise in emerging alien species results from increased accessibility of new source pools. *Proc. Natl. Acad. Sci. USA* **2018**, *115*, E2264–E2273. [CrossRef]
11. Haubrock, P.J.; Turbelin, A.J.; Cuthbert, R.N.; Novoa, A.; Taylor, N.G.; Angulo, E.; Ballesteros-Mejia, L.; Bodey, T.W.; Capinha, C.; Diagne, C.; et al. Economic costs of invasive alien species across Europe. *NeoBiota* **2021**, *67*, 153–190. [CrossRef]
12. Zenni, R.D.; Essl, F.; García-Berthou, E.; McDermott, S.M. The economic costs of biological invasions around the world. *NeoBiota* **2021**, *67*, 1–9. [CrossRef]
13. Medlock, J.M.; Hansford, K.M.; Schaffner, F.; Versteirt, V.; Hendrickx, G.; Zeller, H.; Bortel, W.V. A review of the invasive mosquitoes in Europe: Ecology, public health risks, and control options. *Vector Borne Zoonotic Dis.* **2012**, *12*, 435–447. [CrossRef] [PubMed]
14. Ogden, N.H.; Wilson, J.; Richardson, D.M.; Hui, C.; Davies, S.J.; Kumschick, S.; Le Roux, J.J.; Measey, J.; Saul, W.C.; Pulliam, J. Emerging infectious diseases and biological invasions: A call for a One Health collaboration in science and management. *R. Soc. Open Sci.* **2019**, *6*, e181577. [CrossRef] [PubMed]
15. Kowarik, I. On the role of alien species in urban flora and vegetation. In *Urban Ecology*; Marzloff, J.M., Shulenberger, E., Endlicher, W., Alberti, M., Bradley, G., Ryan, C., Simon, U., ZumBrunnen, C., Eds.; Springer: Boston, MA, USA, 2008; pp. 321–338. [CrossRef]
16. Padayachee, A.L.; Irlich, U.M.; Faulkner, K.T.; Gaertner, M.; Procheş, Ş.; Wilson, J.R.; Rouget, M. How do invasive species travel to and through urban environments? *Biol. Invasions* **2017**, *19*, 3557–3570. [CrossRef]
17. Mayer, K.; Haeuser, E.; Dawson, W.; Essl, F.; Kreft, H.; Pergl, J.; Pyšek, P.; Weigelt, P.; Winter, M.; Lenzner, B.; et al. Naturalization of ornamental plant species in public green spaces and private gardens. *Biol. Invasions* **2017**, *19*, 3613–3627. [CrossRef]
18. Maxwell, B.D.; Lehnhoff, E.; Rew, L.J. The rationale for monitoring invasive plant populations as a crucial step for management. *Invasive Plant Sci. Manag.* **2009**, *2*, 1–9. [CrossRef]
19. Xu, S.Z.; Li, Z.Y.; Jin, X.H. DNA barcoding of invasive plants in China: A resource for identifying invasive plants. *Mol. Ecol. Resour.* **2018**, *18*, 128–136. [CrossRef] [PubMed]

20. Nath, S.; VanSlambrouck, J.T.; Yao, J.W.; Gullapalli, A.; Razi, F.; Lu, Y. DNA barcoding of terrestrial invasive plant species in Southwest Michigan. *Plant Direct* **2024**, *8*, e615. [CrossRef]
21. Ghahramanzadeh, R.; Esselink, G.; Kodde, L.P.; Duistermaat, H.; van Valkenburg, J.L.C.H.; Marashi, S.H.; Smulders, M.J.M.; van de Wiel, C.C.M. Efficient distinction of invasive aquatic plant species from non-invasive related species using DNA barcoding. *Mol. Ecol. Resour.* **2013**, *13*, 21–31. [CrossRef]
22. de Vere, N.; Rich, T.C.; Trinder, S.A.; Long, C. *DNA Barcoding for Plants*; Humana Press: New York, NY, USA, 2015; pp. 101–118. [CrossRef]
23. Hollingsworth, P.M.; Graham, S.W.; Little, D.P. Choosing and using a plant DNA barcode. *PLoS ONE* **2011**, *6*, e19254. [CrossRef]
24. Gorini, T.; Mezzasalma, V.; Deligia, M.; De Mattia, F.; Campone, L.; Labra, M.; Frigerio, J. Check your shopping cart: DNA barcoding and mini-barcoding for food authentication. *Foods* **2023**, *12*, 2392. [CrossRef]
25. Early, R.; Bradley, B.A.; Dukes, J.S.; Lawler, J.J.; Olden, J.D.; Blumenthal, D.M.; Gonzalez, P.; Grosholz, E.D.; Ibañez, I.; Miller, L.P.; et al. Global threats from invasive alien species in the twenty-first century and national response capacities. *Nat. Commun.* **2016**, *7*, 12485. [CrossRef] [PubMed]
26. Wang, X.Y.; Zheng, S.H.; Liu, Y.; Han, J.P. ITS2, a Better DNA Barcode than ITS in Identification of Species in *Artemisia* L. *Chin. Herb. Med.* **2016**, *8*, 352–358. [CrossRef]
27. Chen, S.L.; Yao, H.; Han, J.P.; Liu, C.; Song, J.Y.; Shi, L.C.; Zhu, Y.J.; Ma, X.Y.; Gao, T.; Pang, X.H.; et al. Validation of the ITS2 region as a novel DNA barcode for identifying medicinal plant species. *PLoS ONE* **2010**, *5*, e8613. [CrossRef] [PubMed]
28. Frigerio, J.; Gorini, T.; Galimberti, A.; Bruni, I.; Tommasi, N.; Mezzasalma, V.; Labra, M. DNA barcoding to trace Medicinal Aromatic Plants from the field to the food supplement. *J. Appl. Bot. Food Qual.* **2019**, *92*, 33–38. [CrossRef]
29. Reaser, J.K.; Burgiel, S.W.; Kirkey, J.; Brantley, K.A.; Veatch, S.D.; Burgos-Rodríguez, J. The early detection of and rapid response (EDRR) to invasive species: A conceptual framework and federal capacities assessment. *Biol. Invasions* **2020**, *22*, 1–19. [CrossRef]
30. Venette, R.C.; Gordon, D.R.; Juzwik, J.; Koch, F.H.; Liebhold, A.M.; Peterson, R.K.; Yemshanov, D. Early intervention strategies for invasive species management: Connections between risk assessment, prevention efforts, eradication, and other rapid responses. In *Invasive Species in Forests and Rangelands of the United States: A Comprehensive Science Synthesis for the United States*; Springer: Cham, Switzerland, 2021; pp. 111–131. [CrossRef]
31. Lozano, V.; Marzialetti, F.; Acosta, A.T.R.; Arduini, I.; Bacchetta, G.; Domina, G.; Laface, V.L.A.; Lazzeri, V.; Montagnani, C.; Musarella, C.M.; et al. Prioritizing management actions for invasive non-native plants through expert-based knowledge and species distribution models. *Ecol. Indic.* **2024**, *166*, 112279. [CrossRef]
32. Gaertner, M.; Wilson, J.R.; Cadotte, M.W.; MacIvor, J.S.; Zenni, R.D.; Richardson, D.M. Non-native species in urban environments: Patterns, processes, impacts and challenges. *Biol. Invasions* **2017**, *19*, 3461–3469. [CrossRef]
33. Heringer, G.; Fernandez, R.D.; Bang, A.; Cordonnier, M.; Novoa, A.; Lenzner, B.; Capinha, C.; Renault, D.; Roiz, D.; Moodley, D.; et al. Economic costs of invasive non-native species in urban areas: An underexplored financial drain. *Sci. Total Environ.* **2024**, *917*, 170336. [CrossRef]
34. Frigerio, J.; Campone, L.; Giustra, M.D.; Buzzelli, M.; Piccoli, F.; Galimberti, A.; Cannavacciuolo, C.; Ouled Larbi, M.; Co-lombo, M.; Ciocca, G.; et al. Convergent technologies to tackle challenges of modern food authentication. *Heliyon* **2024**, *10*, e32297. [CrossRef]
35. Layne, T.; Jackson, K.; Scott, A.; Tanner, N.A.; Piland, A.; Haverstick, D.M.; Landers, J.P. Optimization of novel loop-mediated isothermal amplification with colorimetric image analysis for forensic body fluid identification. *J. Forensic Sci.* **2021**, *66*, 1033–1041. [CrossRef]
36. Deliveyne, N.; Young, J.M.; Austin, J.J.; Cassey, P. Shining a LAMP on the applications of isothermal amplification for monitoring environmental biosecurity. *NeoBiota* **2023**, *82*, 119–144. [CrossRef]
37. Deliveyne, N.; Austin, J.J.; Cassey, P. Developing Loop Mediated Isothermal Amplification (LAMP) assays for rapid, presumptive DNA detection of an invasive reptile (Boa constrictor). *Wildl. Res.* **2023**, *51*, WR23053. [CrossRef]
38. Yang, Q.; Domesle, K.J.; Ge, B. Loop-mediated isothermal amplification for Salmonella detection in food and feed: Current applications and future directions. *Foodborne Pathog. Dis.* **2018**, *15*, 309–331. [CrossRef] [PubMed]
39. Lakshmi, B.A.; Kim, S. Recent trends in the utilization of LAMP for the diagnosis of viruses, bacteria, and allergens in food. In *Recent Developments in Applied Microbiology and Biochemistry*; Academic Press: Cambridge, MA, USA, 2021; pp. 291–297.
40. Frigerio, J.; Gorini, T.; Palumbo, C.; De Mattia, F.; Labra, M.; Mezzasalma, V. A fast and simple DNA mini-barcoding and RPA assay coupled with lateral flow assay for fresh and canned mackerel authentication. *Food Anal. Methods* **2023**, *16*, 426–435. [CrossRef]