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Development of an Environmental DNA Assay for Prohibited Matter Weed Amazon Frogbit (*Limnobium laevigatum*)

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Abstract: Environmental DNA (eDNA) is widely used for detecting target species, including monitoring endangered species and detecting the presence of invasive species. Detecting targeted species using the eDNA approach is typically carried out with species-specific qPCR assays. Amazon frogbit (*Limnobium laevigatum*) is classified as a State-Prohibited Matter Weed in NSW, Australia. It is a fast-growing perennial aquatic weed that outcompetes native aquatic plants, leading to a reduction in the habitats of aquatic animals. Early detection is crucial for the effective management of this species. In this study, we developed a qPCR assay for *L. laevigatum* based on the *rpoB* gene sequence. This assay was validated against 25 non-target aquatic and terrestrial species. It was found to be species-specific, with the positive signal exclusively detected in *L. laevigatum*. The assay was highly sensitive with the modelled detection limits of 3.66 copies of DNA/µL. Furthermore, our assay was validated using environmental samples collected from field sites with and without the presence of *L. laevigatum*. Our assay is an effective tool for targeted eDNA detection of *L. laevigatum*, which will enhance efforts to monitor and control this invasive aquatic weed.

Keywords: invasive plants; eDNA; biosecurity; quantitative PCR; aquatic weeds

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1. Introduction

Environmental DNA (eDNA) refers to genetic materials obtained from environmental samples without information about the source organisms [1]. Since the first application of eDNA in macro-organisms in 2005 [2], it has been widely used for monitoring species in various environments, particularly in aquatic ecosystems [3,4]. EDNA technologies offer a non-invasive, sensitive and cost-efficient approach compared with traditional labor-intensive surveys.

EDNA has great potential for biosecurity surveillance of non-indigenous species (NIS), which are often rare and challenging to detect through visual observation. Nowadays, eDNA has been extensively applied to monitor target and non-target NIS using qPCR [4], digital PCR [5], or metabarcoding [6]. Although metabarcoding allows the simultaneous monitoring of multiple NIS, several studies reported that qPCR and digital PCR methods could provide higher sensitivities [7–10]. In addition, metabarcoding often faces challenges identifying plant DNA to the species level due to general lack of a single universally informative gene target suitable for plant DNA barcoding, and genetic overlap among some species at targeted genes [11].

Despite being broadly applied in monitoring of animal species, the application of eDNA in weed biosecurity is limited [12,13]. Several studies used a metabarcoding approach for biodiversity analyses to investigate the plant communities using soil [14], dust [15] and honey [16] samples. Additionally, qPCR-based targeted species detection methods have been developed for various aquatic weeds, such as *Hydrilla verticillata* (L.f.)

Royle, *Egeria densa* Planch. and *Myriophyllum spicatum* L. [17–19]. These studies suggest that eDNA is a useful tool in addition to the traditional monitoring approaches for weed biosecurity.

Limnobium laevigatum (Humb. & Bonpl. ex Willd.) Heine, Amazon frogbit, is listed as a Prohibited Matter Weed in New South Wales (NSW) [20]. Native to Central and South America [21,22], this species has been widely introduced beyond its native range. *Limnobium laevigatum* is recognised as a noxious weed in numerous countries, including Australia, Indonesia, Japan, USA, Zambia and Zimbabwe. It was sold as an ornamental aquatic plant in Australia and first found outside of cultivation in 2011 in Queensland (QLD), and has since been reported in NSW, Western Australia (WA), Northern Territory (NT), and Victoria (VIC).

Limnobium laevigatum is a fast-growing, freshwater, perennial species that outcompetes native aquatic plant species, obstructs waterways and reduces shelter for aquatic animals [20]. *Limnobium laevigatum* is adapted to grow in tropical and subtropical climates [20]. It grows on the surface of freshwater, either free-floating or with its roots anchored in underwater substrates [20]. *Limnobium laevigatum* reproduces sexually through seed and asexually through stolon segments. Each plant can produce multiple seed pods, with each pod containing 20–30 seeds [20]. The seed bank can last for at least three years. The floating rosettes and seeds can be easily dispersed by water [20].

Currently, surveys for new incursions of *L. laevigatum* in Australia rely heavily on labour-intensive visual detection with the assistance of weed officers, contractors and volunteers. New approaches, such as remote sensing, are often limited by heavy canopy coverage and expensive operational costs. Visual monitoring of *L. laevigatum* is further hindered by the presence of a morphologically similar aquatic plant, *Hydrocharis dubia* (Blume) Backer, also commonly known as frogbit.

The use of eDNA offers an approach for early detection of weed species across all stages of their development. By so doing, it can enable proactive management efforts to identify and control novel weed emergences or, alternatively, monitor the spread of those that have established. In this study, we aimed to develop species-specific qPCR assays for *L. laevigatum* and validate the assays using environmental samples. Our goal is to establish a robust protocol for the detection and monitoring of *L. laevigatum.* The developed protocol will enhance weed management strategies and contribute to the preservation of aquatic ecosystems.

2. Materials and Methods

2.1. Specimens

The plant tissues used in this study comprised herbarium and fresh samples, including *L. laevigatum* (N = 16) from various localities and 25 non-targeted species (N = 64) used as negative controls in specificity testing of the assay (Supplementary Table S1). DNA extraction from sampled specimens was carried out using DNeasy Blood and Tissue Kit (Qiagen, Hilden, Germany), following the manufacturer's instructions. The species identity of each sample was genetically verified through their *rbcL* sequences (Supplementary Table S1). The sequences of these specimens were deposited in GenBank under accession numbers PP001306-PP001385.

2.2. Assay Development

We obtained chloroplast genomic sequences of *L. laevigatum* and closely related species from GenBank. The sequences were aligned using MAFFT [23] and manually adjusted using BioEdit [24]. We selected highly diverse gene regions for designing primers and probes.

For *L. laevigatum*, we designed the diagnostic assay Ll26823. The assay targeted a 125 bp region of the chloroplast *rpoB* gene. This region genetically differs by over 12% between *L. laevigatum* and its most closely related species in Australia, *H. dubia* [25] (Figure 1). Primers and probes (Table 1) of assay Ll26823 were visually designed, and we used Multiple Primer Analyzer [26] to check them for the potential presence of confounding secondary

structures. The primers and probes were synthesised by Sigma Aldrich (Woodlands, USA) with HPLC purification. G-Block DNA was synthesised by Integrated DNAs Technologies (Coralville, USA; Table 1) and used for assay optimisation.

Table 1. Primers and probe designed for *Limnobium laevigatum*, amplifying a 125 bp region of the *rpoB* genes. T_m, melting temperature.

Label	Direction	Sequence (5′-3′)	T_m (°C)
LlF26823	Forward	AAGGGGTGATATTTTAGTAGGC	59.0
LlR26823	Reverse	AGCAAGTTTCTTTCGTAACG	58.7
LlP26823	Probe	[6FAM] CTCAGGAAACGAGCGAATTATCCTATG [BHQ1]	68.9
Ll26823gBlock		GGGTAGAAAGGGGTGATATTTTAGTAGGCAAATTAACACCTCAGGAAACGAGCG AATTATCCTATGCTCCGGAGGATAGATTATTACGAGCCATACTTGGCATTCAGGTA TCCGTTACGAAAGAAACTTGCTTAAAA	

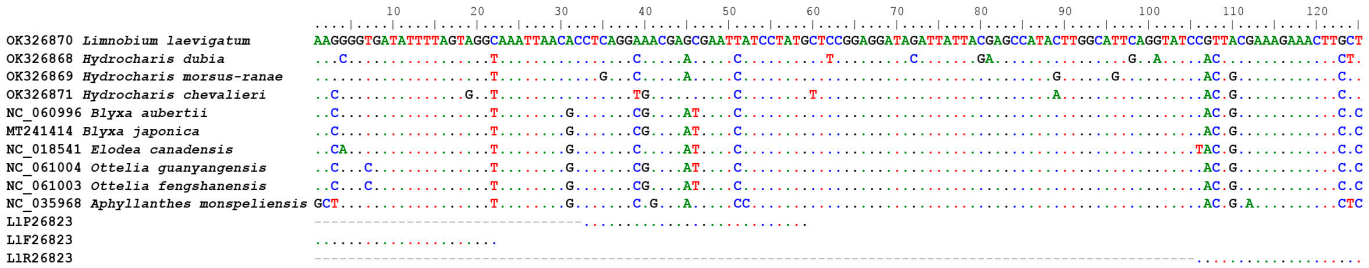

Figure 1. Primer annealing sites in *Limnobium laevigatum*, targeting the *rpoB* gene.

We optimised and tested the Ll26823 assay using the synthetic gBlock DNA. A standard curve of the Ll26823 assay was calculated using 10-fold serial dilutions of the gBlock, ranging from 10^6 copies/µL to 1 copy/µL with three replicates per dilution. The qPCR reactions were conducted in a 10 µL solution, consisting of 1 × PerfecTa Tough Mix (QuantaBio, Beverly, MA, USA), 900 nM of each primer, 250 nM of the probe and 2.5 µL of DNA template. The qPCR runs were performed on a Magnetic Induction Cycler (MIC, Bio Molecular Systems, Upper Coomera, Australia) with an initial denaturation for 5 min at 95 °C and 40 cycles of denaturing at 95 °C for 30 s and annealing at 58 °C for 30 s. We conducted an additional eight qPCR replicates using gBlock DNA at 5, 2.5, 1 and 0.5 copies/µL and evaluated the limits of detection (LOD) and quantification (LOQ) of the Ll26823 assay using the R script described by Klymus, et al. [27]. The specificity of the assays was tested using replicates of *L. laevigatum* and 25 non-targeted species (Supplementary Table S1).

2.3. Assay Multiplexing with QC1292

QC1292 is a quality control qPCR assay designed to target generic plant and bacterial DNA in the environment and can be used as a control to monitor the entire eDNA workflow [28]. We multiplexed Ll26823 with QC1292 to detect any potential false negatives resulting from processing errors and/or PCR inhibitors. The efficiency and sensitivity of the multiplexed assays were evaluated using a 1:1 mixture of gBlock DNA of the Ll26823 and the QC1292 assays. The gBlock DNA concentrations and qPCR reactions were conducted under the same reaction conditions we reported for the singleplex reactions with the addition of 100 nM of forward and reverse QC1292 primers and 28 nM of the QC1292 probe.

2.4. Field Application

To evaluate the applicability of these assays to field samples, we tested freshwater environmental samples collected from various locations in NSW, including locations with active *L. laevigatum* infestations, historical *L. laevigatum* infestations, and locations without historical records of *L. laevigatum* (Table 2 and Supplementary Table S2). Sample sites from Park Road, Bellambi, were located 500 m upstream from the infested property. Samples

from Cawley Street, Bellambi, and Bellambi Lagoon were located 150 and 500 m downstream of the infested property, respectively (Supplementary Table S2). At most locations, samples were collected using a Smith-Root eDNA sampler with a self-preserving 5 µm PES filter unit (EnviroDNA, Brunswick, Victoria, Australia). Up to 2.0 L of water was filtered for each sample, with a targeted flow rate of 1.0 L/min, minimum flow rate of 0.3 L/min, and pressure limit of 10 psi. Two to ten samples were collected from each location, depending on the size of the water body and the volume of water that could be filtered (Supplementary Table S2). Prior to sampling at each location, a negative control was obtained by filtering 1.0 L ultrapure water using the Smith-Root eDNA sampler, as described above. Filters were stored at room temperature until returning to the laboratory, where they were removed from the filter housings and cut in half. One half was stored at −20 °C for later use, while the other half was cut into approximately 5 mm squares, and the eDNA was extracted using DNeasy Blood and Tissue Kit (Qiagen, Hilden, Germany) as described [28].

Table 2. Results of analysis with the field-collected eDNA samples using the *Limnobium laevigatum* qPCR assay Ll268233 multiplexed with the QC1292 assay. All values are shown as mean ± standard error of the mean. Further details of these environmental samples are included in Supplementary Table S2. N/A: No Cq or eDNA concentration was recorded when there was no amplification in the Ll268233 assay for any sample or replicate.

Locality	Cq ± SE *	eDNA Concentration ± SE in the Sampled Environment **
Commercial Road, Oakville	28.73 ± 1.21	$4.09 \times 10^5 \pm 1.88 \times 10^5$
Ogden Road, Oakville	23.66 ± 0.21	$1.11 \times 10^6 \pm 7.84 \times 10^4$
Bellambi Lagoon	34.63 ± 0.49	$4.48 \times 10^3 \pm 6.35 \times 10^2$
Cawley Street, Bellambi	31.71 ± 0.64	$9.09 \times 10^4 \pm 4{,}53 \times 10^4$
Edyth Street, Corrimal	31.43	$1.92 \times 10^4 \pm 1.92 \times 10^4$
Park Road, Bellambi ***	N/A	N/A
Bulahdelah ***	N/A	N/A
Rileys Creek, Catherine Field ***	N/A	N/A
Lagoon, Catherine Field	25.06 ± 0.88	$1.06 \times 10^6 \pm 3.37 \times 10^5$
Bomaderry	30.30 ± 0.26	$3.20 \times 10^5 \pm 1.56 \times 10^5$
Lake Albert, Wagga Wagga ***	N/A	N/A

* Mean Cq value ± standard error of mean (SE). Samples and replicates failing QC or with a negative Ll268233 assay are excluded. ** Samples and replicates failing QC are excluded. Samples and replicates with a negative Ll268233 assay are considered to have 0 eDNA copies/L of water filtered. *** location with no history of *L. laevigatum*.

Samples from Bulahdelah were obtained using a syringe-based eDNA sampling kit with a 5 µm PES filter unit (EnviroDNA, Brunswick, Victoria, Australia) following the manufacturer's instructions. Two replicates and a negative control were collected. In the laboratory, 20 µL of proteinase K (20 mg/mL, Qiagen, Hilden, Germany) was added directly to the syringe filter unit without removing the preservative buffer and incubated overnight at 56 °C with the syringe attached. After incubation, the syringe was used to remove the lysate from the filter, and eDNA was extracted from the lysate using the DNeasy Blood and Tissue Kit. Each sample was tested using multiplex Ll26823 + QC1292 assays using the aforementioned reaction systems with three technical replicates. The individual qPCR efficiency [29] of each reaction was calculated using the built-in software in MIC. The concentration (copy number/µL) of DNA extractions was calculated from the standard curve generated by qPCR of gBlock, using the software in MIC. Samples that yielded a negative result in the QC1292 assay or had an efficiency lower than 85% in all qPCR replicates were excluded from the analysis. The concentration (copy number/L) of the eDNA in the environment was calculated using the formula (a × b)/(c × d), where:

a represents the eDNA concentration of the DNA extraction (copy number/µL),
b denotes the elution volume of DNA extraction (µL),
c is the total volume of water filtered for each sample (L), and

d indicates the proportion of filter extracted.

3. Results

3.1. Assay Development

Assay Ll26823 (Table 1) was developed based on a region of the chloroplast coding gene *rpoB*, and this region exhibited high levels of genetic variation among closely related species (Figure 1). The assay was specific to its target species and successfully amplified all 16 *L. laevigatum* samples in all replicates, while all 25 non-target species yielded negative results in the qPCR.

3.2. Assay Sensitivity

Synthetic gBlock DNA was used to evaluate the sensitivity of the Ll26823 assay. Our assay demonstrated high efficiency and sensitivity (Table 3). The qPCR efficiency of the Ll26823 assay was 99.06% when run as a singleplex reaction. When multiplexed with quality control assay QC1292, the Ll26823 assay maintained a high qPCR efficiency at 94.54%. The modelled limit of detection (LOD) and the limit of quantification (LOQ) of Ll26823 changed from 3.66 and 86 copies/µL when singleplexed to 16.5 and 16.5 copies/µL when multiplexed, respectively (Table 3).

Table 3. The performance of the species-specific assay of *Limnobium laevigatum* when run as singleplex or multiplex (*) reactions. Showing the efficiency, modelled limit of detection (LOD) and modelled limit of quantification (LOQ) of Ll26823.

Assay	Target	Efficiency (%)	LOD (Copies/µL)	LOQ (Copies/µL)
Ll26823	*L. laevigatum*	99.06	3.66	86
Ll26823 + QC1292 *	*L. laevigatum* and endogenous DNA	94.54	16.50	16.50

QC1292 is a positive control qPCR targeting endogenous DNA used to monitor the entire eDNA workflow [28].

3.3. Field Application

The field application of multiplexed Ll26823 and QC1292 assays successfully detected *L. laevigatum* in all samples collected from the sites where the species was recorded in the three months prior to sampling, with Cq values ranging from 22.47 to 31.43 and eDNA concentrations from 1.92×10^4 to 1.11×10^6 copies/L (Table 2). The assay also successfully detected *L. laevigatum* at approximately 150 m and 500 m downstream from a source infestation (Supplementary Table S2). With increasing distance, the mean Cq value increased from 31.71 to 34.63, and eDNA concentrations decreased from 9.09×10^4 to 4.48×10^3, respectively. In contrast, no *L. laevigatum* eDNA was detected in the negative controls, at the control sites where the species had never been recorded, at one historic site where *L. laevigatum* had not been recorded for eight months prior to sampling, or upstream from an infestation (Table 2 and Supplementary Table S2). Endogenous DNA was detected in all environmental samples with individual qPCR efficiency over 85%, except as indicated in Supplementary Table S2, suggesting the absence of systematic errors in the eDNA workflow and minimal inhibition of the reaction.

4. Discussion

In this study, we developed a qPCR assay for the detection of *L. laevigatum* eDNA. The assay targets the chloroplast *rpoB* gene, which is normally present in hundreds to thousands of copies in plant leaf cells. Given the high sensitivity of this assay, we expect that it will assist in *L. laevigatum* monitoring for early detection of the weed and confirmation of eradication.

EDNA has been widely applied to animal and insect monitoring for biosecurity [30] and conservation purposes [31]. However, the application of eDNA in plant biosecurity is less common and poses additional challenges. Due to the highly fragmented nature of

eDNA, the target gene region must be short, usually less than 280 bp [1]. A high level of between-species genetic diversity is needed in this target fragment for species-specific analysis, not only for molecular diagnostics but also for assay development. However, there is no generic gene region for plant molecular identification. The *rbcL* and *matK* genes commonly used for plant DNA barcoding are usually not diverse enough for species-specific primer and probe design. In the case of *L. laevigatum*, the genetic distance to the closest species, *H. dubia*, in Australia is 95.6% and 98.9% for *matK* and *rbcL* genes (Based on Blast result of sequences AB002574 and NC_061221, and AB004894 and AB004892), respectively. It is very difficult to design a species-specific qPCR assay based on these low levels of genetic distance. Sequence information, especially for some weed species, is limited in public databases, such as GenBank. *Limnobium laevigatum*, for example, only has 11 sequences available in GenBank (accessed 26 December 2023). It is challenging to find a gene region that has been sequenced among closely related species and is sufficiently diverse for assay development. In this study, we compared the chloroplast genome of *L. laevigatum* against other closely related species from the Family of Hydrocharitaceae [25] and discovered the highly diverse *rpoB* gene (Figure 1). As a coding region, this region is generally more conserved than non-coding regions within the same species. Subsequently, intraspecific polymorphisms in the gene are rare, and chances of obtaining false negatives due to primer and or probe mismatch are low.

The amount of DNA released from an individual plant can vary significantly depending on its developmental stages, and this can present a challenge to the detection of plant eDNA [19]. Kuehne, et al. [19] reported very low eDNA concentrations of *Egeria densa* and *Myriophyllum spicatum*. The amount of eDNA detected only weakly corresponded with plant abundance but increased significantly during plant senescence [19]. Often, due to the release of pollen, the observed plant eDNA peaks during the flowering period. Surprisingly, Kuehne, et al. [19] did not report such a peak in their study. Other research has reported higher eDNA concentration during the growth period of *Hydrilla verticillata* [32].

In our study, we did not measure the relationship between eDNA abundance and the *L. laevigatum* growth stage or its biomass. Unlike biodiversity conservation research, biosecurity research targets NIS, in this case, a Prohibited Matter Weed, which is required to be eradicated from the environment. The detection of the species triggers the same management responses regardless of how many plants are found. Similarly, the detection of eDNA raises the alarm that the target species could potentially be present in the environment. Paired with research on eDNA degradation (research in progress), we anticipate that future studies may enable us to use eDNA abundance to estimate the potential distribution of *L. laevigatum* at various spatial scales, as well as determine the frequency of field surveys required.

In this study, we detected *L. laevigatum* from all locations where the species was recently recorded. Compared with other studies of aquatic plants based on qPCR of cpDNA markers, the eDNA concentration detected in our study was similar to that measured earlier, ranging from 4.48×10^3 to 1.11×10^6 copies/L. This compares with a maximum of around 3.5×10^4 copies/L of invasive *Egeria densa* in Japan [33] and up to 2.12×10^5 copies/L of the native *Hydrilla verticillata* in Japan [17]. We found a decrease of more than an order of magnitude between samples taken ~150 m and ~500 m downstream from an infestation and no detectable eDNA upstream from an infestation. This suggests that eDNA samples taken at regular intervals along a watercourse or riverine drainage system could be used to more effectively direct manual surveillance efforts. For instance, if downstream eDNA is detected, focusing upstream surveillance efforts may reveal sites likely containing patches of an invasive weed. The relationships between eDNA concentration and plant abundance or biomass, and between eDNA concentration and distance from source plants, is poorly understood (see above), so this may need to be assessed on a species-by-species basis. Concentrations of eDNA may be influenced by variables such as plant developmental stage, with some studies showing a peak in eDNA concentration during plant senescence [19,33] and others showing a peak during the growth period [32]. Given that all the sample sites in

our study had been controlled with herbicides, we may expect high eDNA concentrations due to forced "senescence", a result of the control efforts. Further studies under controlled laboratory conditions, as well as repeated sampling from the same field sites over time, would help to parameterise relationships between eDNA concentration, plant abundance, and growth stage for *L. laevigatum*.

It's worth mentioning that eDNA of *L. laevigatum* was detected not only in the locations where live plants were visually observed but also in previous infestation sites where no live plants were observed. For example, we detected *L. laevigatum* eDNA at Ogden Road, Oakville, where the plans were most recently observed 3 months earlier. This finding highlighted an important issue of legacy DNA, which can persist in the environment for a very long period under optimal conditions after the source species have been extirpated, e.g., ancient DNA. The persistence of legacy DNA in the environment is a case-by-case issue depending on the type of environment, temperature, pH, target species, etc. [34–37]. Therefore, the detection of eDNA does not necessarily equate to the presence of target species. This issue could possibly be resolved with environmental RNA (eRNA), which degrades much faster than eDNA [37]. A positive detection of eRNA is normally considered to be the detection of a living target [38]. However, the nature of rapid degradation poses a considerable challenge in eRNA research, which is currently much less studied compared with eDNA. The eRNA analysis for *L. laevigatum* is currently under development, which could potentially further assist in the monitoring of *L. laevigatum* and eliminate the potential issue of false positives caused by the persistence of legacy DNA in the environment.

Notably, in our study, we incorporated a quality control qPCR QC1292 in the assay to monitor false negative results [28]. The eDNA workflow consists of multiple steps, from sample collection to data analysis [39]. A negative result could arise not only from the absence or low abundance of the target DNA but also from systematic errors in the workflow, such as a broken filter. The introduction of a quality control assay targeting endogenous eDNA enables the monitoring of the entire workflow and enhances the reliability of interpreting the eDNA results. In addition, the target assay Ll26823 exhibited a similar LOD when multiplexed with the quality control assay, which was similar to a previous study [28].

In this study, we demonstrate that *L. laevigatum* infestations can be detected through eDNA using a qPCR assay. Further research using this assay under controlled laboratory conditions, as well as the development of eRNA methods, will improve our understanding of the detectability of *L. laevigatum* in the field. This will provide a useful surveillance tool for delimitation of new infestations of *L. laevigatum* and for assessment of the effectiveness of eradication efforts.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/environments11040066/s1, Table S1: Plant samples used in this study for species specification test of Ll26823 assay. Table S2. Details of environmental samples collected in this study. N: number of replicates per location.

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