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Development of a real-time PCR assay for the detection of the golden mussel (*Limnoperna fortunei*, Mytilidae) in environmental samples

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ABSTRACT

The golden mussel, *Limnoperna fortunei*, is among the most devastating invasive species in freshwater habitats worldwide, leading to severe environmental disturbances and economic losses. Therefore, management efforts would be greatly improved by methods that efficiently detect and quantify the abundance of the golden mussel in freshwater habitats, particularly in early stages of colonization. In this study, we describe a highly-sensitive real-time PCR assay targeting a 100-bp region of the COI mitochondrial gene of the golden mussel. The method was able to detect as little as 0.225 pg of target DNA. This assay represents an important contribution to surveillance methods, as well as to optimize field measures to contain and manage populations of the golden mussel in its introduced range.

Key words: eDNA, invasive species, environmental DNA, molecular identification.

INTRODUCTION

The golden mussel *Limnoperna fortunei* (Mytilidae, Bivalvia) is an invasive bivalve from Southeast Asia rivers (Xu et al. 2015). Due to the characteristics of its reproductive cycle and its high adaptability to different environmental conditions, its great phenotypic plasticity, high fecundity and byssal attachment to different substrates (Iummato et al. 2013), the golden mussel is among the most important invasive species in continental waters worldwide. *Limnoperna fortunei* is a very effective ecosystem engineer, altering both ecosystem structure and function, and causes great ecological and economic impacts (Darrigran and Ezcurra-de-Drago 2000, Boltovskoy 2015). This species has also invaded hydraulic structures in South America and other Asian countries causing serious biofouling problems (Boltovskoy et al. 2006)

The efficiency of methods for the management and control of the golden mussel depends heavily on the ability of detecting its presence, particularly

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in the early stages of its colonization. To this end, Pie et al. (2006) developed a set of species-specific primers that could be used to detect golden mussel larvae obtained from filtered environmental water. Although the method is clearly an advantage over the laborious and error-prone approach of screening larvae under the microscope, its application is limited by three main factors. First, large volumes of water had to be filtered (typically > 500 L), which is a logistic challenge, particularly in remote areas or in turbulent waters. Second, the method only provides binary information in terms of presence or absence of the golden mussel in the obtained samples. Finally, the large fragment that was amplified (~ 300 bp) requires that the template DNA is relatively well preserved, allowing for its detection only in the presence of fresh larval tissue in the samples. However, recent studies have sought to detect free DNA molecules in suspension, also known as eDNA, which usually involves smaller fragments (Ficetola et al. 2008, Taberlet et al. 2012). eDNA methods have been particularly useful in monitoring programs of invasive species, including frogs (Ficetola et al. 2008), fish (Darling and Mahon 2011, Keskin 2014) and mudsnails (Goldberg et al. 2013).

In this study we describe a highly-sensitive real-time PCR assay targeting a 100-bp region of the COI mitochondrial gene of the golden mussel. Real-time PCR is a powerful technique that has been used in a variety of applications, including genotyping, gene expression analyses, and pathogen detection (Mackay 2007). In this method, fluorescence data are obtained during the logarithmic phase of the PCR, when the quantity of the amplified product is directly proportional to the amount of initial template, thus circumventing the need for post-PCR processing. Fluorescence is provided by a specific probe, which provides a second layer of specificity in addition to the method (Dias et al. 2008). As a consequence, rtPCR is faster and more accurate than its traditional alternatives (Wilcox et al. 2013, Nathan et al. 2014). The availability of this new assay could represent an important contribution to improve surveillance methods, as well as to optimize field measures to contain and manage populations of the golden mussel in its introduced range.

MATERIALS AND METHODS

PRIMER DESIGN

We downloaded 57 sequences of the cytochrome c oxidase subunit I (COI) mitochondrial gene of the golden mussel available on genbank, particularly those sequenced by Ghabooli et al. (2013) and searched for conserved regions that would allow for the amplification of a short fragment that could be used even in the case of highly degraded environmental DNA. In addition, we targeted a mitochondrial region because there are more mitochondrial DNA copies per cell than nuclear DNA copies, thus maximizing the chance of detection. A set of primers and a probe for a TaqMan® assay were designed using PrimerQuest. To ensure the specificity of the probe, we checked for potential cross amplification using BLAST (Altschul et al. 1990). The probe was synthesized with a 6-GAM reporter dye in the 5’- end and MGB-NFQ as a quencher on the 3’- end.

Sensitivity of the real-time PCR assay was assessed with genomic DNA obtained from adult muscle tissue from *Limnoperna fortunei* extracted using DNeasy® Blood & Tissue Kit (Qiagen). Assays were carried out using a StepOnePlus™ Real-Time PCR System (Applied Biosystems). The test was performed in a series of six 1:10 dilutions from the initial 150 ng/µl of DNA template. Generally the concentrations from eDNA extractions are covered from the spectrum of these dilutions (Spear et al. 2015). To ensure the results, the extractions and PCR were carried out in separate rooms. As commonly used in this kind of experiment to certify the test credibility, a list of controls were

performed: a standard negative control, a negative control with DNA from a knowing different species and TaqMan® Exogenous Internal Positive Control (Applied Biosystems). Finally, to test for PCR inhibition, we included the amplification of the internal positive control as one of the samples.

Total optimized solution was prepared on a 12.5-uL volume including 6.25 uL of TaqMan® Environmental Master Mix 2.0 (Applied Biosystems), 3 µL of DNA as template, 10 nM of each primer, 25 nM of probe, 0.3 µL of TaqMan® Exogenous Internal Positive Control 10X Exo IPC Mix (Applied Biosystems), 0.15 µL of TaqMan® Exogenous Internal Positive Control 50X Exo IPC DNA (Applied Biosystems) and 2.8 µL of Ambion® DEPC-treated water (Ambion®). All samples ran in triplicate for templates and negative controls. The reaction was performed with 95°C for 15 min followed by 40 cycles of denaturation at 94°C for 1 min and annealing/extension at 65°C for 1 min.

Finally, we tested the performance of the method under field conditions by collecting 100 mL water samples from reservoirs of electric power plants in the state of Paraná for which the golden mussel was recorded as either present (Usina Salto Osório - São Jorge D'oeste, and Usina Governador José Richa - municipality of Capitão Leônidas Marques) or absent (Usina Salto Santiago - municipality of Saudade do Iguaçu, Usina Governador Bento Munhoz da Rocha Netto - municipality of Pinhão, and Usina Governador Ney Aminthas Braga - municipality of Mangueirinha). This classification, in turn, was defined either based on previous studies by our own team and through confirmatory analyses based on the collection of environmental samples and screening larvae under the microscope. Each sample was filtered using a 47 mm diameter and 45 μm pore size nitrocellulose membrane. Total DNA was extracted using a DNeasy® Blood & Tissue (Qiagen) kit, following the manufacturer's instructions, using the whole filter rolled up in the microtube for digestion.

RESULTS

The designed real-time PCR assay primers and probe are shown in Table I. The *in silico* analysis using NCBI blast showed that the primers did not match other bivalve species. The assay amplifies a 100 bp region of the COI gene, corresponding to coordinates 316-412 of the matching gene in *Crassostrea angulata* (AB904890). There was a linear range of detection over five orders of magnitude, with little variation among replicates (Figure 1).

TABLE I
Sequences of primers and probe used in our assay for the detection of the golden mussel.

Name	Sequence (5' -3')
Forward primer	GGGACTGGTTGGACAGTTTAT
Probe	6FAM-CCCAGCAGTTGACATAGCTGCTTT-MGB-NFQ (Sense)
Reverse primer	ACGCACCAGCTAAATGAAGA

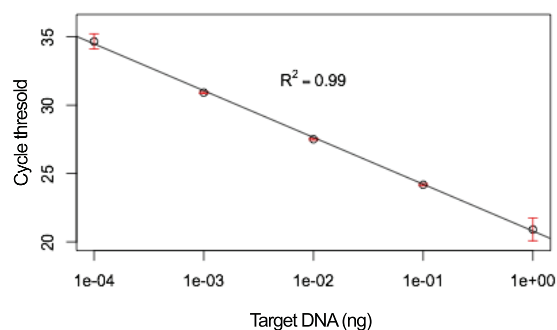

Figure 1 - Sensitivity of the *Limnoperna fortunei* real-time PCR assay. Dilutions were made from genomic DNA and mean values of cycle threshold (Ct) plotted against log_{10}-transformed DNA concentrations. Error bars represent standard deviations over three replicates.

Field tests of the protocol described in this study were highly efficient, given that none of the locations where the golden mussel had not been recorded were tested positive, whereas the method indicated the presence of the golden mussel in both of the reservoirs where populations had been detected.

DISCUSSION

The most crucial moment for the management of invasive species is precisely that in which it is less detectable, i.e. during the early stages of colonization. Therefore, efficient efforts to monitor and control the golden mussel depend heavily on a means for its early detection. In this study we provide a new TaqMan® rtPCR assay that can be used for this purpose. Future tests under field conditions should allow for assessing the extent and operational conditions under which the quantitative information provided by the assay can translate into reliable estimates of golden mussel biomass, as has been proposed for other organisms (e.g., Takahara et al. 2012, Goldberg et al. 2013, Pilliod et al. 2013).

An alternative to the method presented in this study has been designed by Endo et al. (2009), in which a SYBR-Green assay was developed to amplify a 139 bp fragment of the golden mussel COI gene. Given that our primer set would amplify a fragment that is nearly 40% shorter than that designed by Endo et al. (2009), we expect that our method will be more sensitive and robust to template degradation, as commonly observed in environmental DNA. In addition, the use of the TaqMan® probe provides an additional level of specificity in our assay in relation to an alternative SYBR-Green assay.

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