A fast and accurate molecular method for the detection of larvae of the golden mussel *Limnoperna fortunei* (Mollusca: Mytilidae) in plankton samples

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The golden mussel (*Limnoperna fortunei*, Mytilidae) is native to continental China, but is emerging as an important invasive species. It was first recorded in South America in 1991 in the estuary of the Rio de La Plata and dispersed into the largest rivers systems of the Plata basin (Rio de la Plata, Rio Paraná, Rio Uruguay and Rio Paraguay), travelling inland at an estimated rate of 240 km per year.[1]

Economic losses due to invasive mollusc species can be severe, costing the US economy alone more than 1 billion dollars annually.[2] Common effects of these organisms include the biofouling of water intakes of industrial, electric power and drinking water plants, often requiring the use of chemical and mechanical removal methods for their control. Their impact on hydroelectric power plants is of particular concern, given that effects such as the reduction in the diameter of pipes, their clogging, and the accumulation of empty shells translate into immediate economic losses.[3] The deleterious impact of *L. fortunei* in natural environments is also severe. Darrigran *et al.*[4] showed that the golden mussel is displacing native species, as well as allowing the proliferation of uncommon ones.

Efficient management of *L. fortunei* in its introduced range requires the ability to predict sites most likely to experience colonization, permitting targeting of control measures. This goal can be achieved by monitoring the presence of larvae of *L. fortunei* in plankton samples. In principle, this could be done using light microscopy. However, such strategy would be prohibitively time-consuming and inaccurate, given that the identification of planktonic larvae is a notoriously difficult task. Fortunately, molecular methods for the identification of mussel larvae have been developed over the past decade. For instance, immunological techniques have been used to identify scallop larvae (e.g. Paugam *et al.*[5]), although environmental conditions, ontogenetic changes in the larvae, and sample preservation status may alter the antibody binding response.[6] Alternatively, larvae of several mussel species have been efficiently discriminated using restriction fragment-length polymorphism (RFLP[7–9]). This approach is based on the amplification of a DNA fragment of the species of interest and its digestion using restriction enzymes to generate species-specific banding profiles. An important disadvantage of RFLP methods is the need to isolate individual larvae prior to the analysis to avoid co-amplification of different species and therefore would not be ideal for extensive monitoring programs. These limitations have been overcome by the development of species-specific primers.[10,11] In this method, even though the DNA of a variety of species will be present in a given reaction, the designed primers will only anneal to the DNA of the target species. In this paper we describe the design of a set of primers that can be used to detect larvae of the golden mussel.

A fragment of the cytochrome oxidase subunit 1 gene (COI) of *L. fortunei* was sequenced. Total genomic DNA was extracted from a muscle sample using the EZ-DNA kit (Biosystems, Brazil) following manufacturer's instructions. This fragment was amplified using the LCO - HCO universal primer pair (Table 1) with the following PCR protocol: 5 min at 95°C/35 cycles of 92°C for 30 s, 48–51°C for 30 s and 68°C for 30 s/68°C for 2 min in 25 μl reactions with 1.25 units of AmpliTaq DNA polymerase, 1X PCR buffer, 2 mM of $MgCl_2$, 0.4 mM of dNTPs and 0.5–1 μM of each primer. Unique regions were identified by comparing the *L. fortunei* sequence with a sample of 4 mollusc species. Sequences of *Crassostrea gigas*, *Corbicula fluminea*, an unidentified unionid and *Modiolus brasiliensis* were obtained using the same protocol as described above (GenBank Accession numbers DQ264392–DQ264395). Sequence alignment was first obtained using ClustalX[12] and adjusted by eye. The identification of unique regions was based on the frequency of mismatches between *L. fortunei* sequences and the remaining species, giving higher weight to transversions and gaps. A primer pair was designed that should only amplify *L. fortunei* DNA (Table 1). In addition, a second primer pair was used in the same reaction to amplify a region of the 18S ribosomal gene to serve as a positive control. Thus, given that the 18S primers are universal, a PCR amplification of a sample of plankton that lacked *L. fortunei* would only generate the 18S band, but not the specific band. The PCR protocol to obtain specific bands consisted of 4 min at 94°C/32 cycles of 94°C for 30 s, 58.2°C for 30 s and 70°C for 60 s/70°C for 3 min in 25 μl reactions with 0.625 units of AmpliTaq DNA polymerase, 1X PCR buffer, 1.5 mM of $MgCl_2$, and 0.5 mM of dNTPs. Primer concentrations for the 18S and the COI primers were 0.8 and 4 μM, respectively. PCR products were electrophoresed on a 2% agarose gel.

Sensitivity analyses were carried out with increasingly smaller amounts of template DNA to determine the extent of detectability of the method. Finally, specificity tests were conducted by testing the designed primers against samples of the four taxa used for their development, as well as a crustacean (*Ucides cordatus*, Decapoda) and six additional molluscs (*Modiolus brasiliensis*, *Perna perna*, *Colisella* sp., *Littorinidae* sp., *Brachidontes* sp., *Thais* sp.).

The method is very sensitive, detecting DNA amounts smaller than 0.041 ng (Fig. 1). It is important to note that an extraction from a single larva of *L. fortunei* yielded 28.5 ng/μl of DNA, more than 600 times the detectability threshold of our primers. Moreover, the specific band was detected consistently in golden mussel samples, but was never present when using DNA from other species in amplifications using the specific COI primers. Additional tests (not shown) using known concentrations of environmental plankton samples and increasingly smaller amounts of golden mussel DNA showed similar performance. These results indicate that the primer sets provided in this study can become an important tool for testing for the presence of larvae of *L. fortunei* in its introduced range. In fact, this method is already being used to monitor the presence of the golden mussel in the Rio Iguaçu basin. Monthly tests of

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Table 1. List of primers used in the present study.

Primer	Sequence	Gene	Primer type
LCO	5′-GGTCAACAAATCATAAAGATATTGG-3′	COI	Universal
HCO	5′-TAAACTTCAGGGTGACCAAAAATCA-3′	COI	Universal
Limno. COIR1	5′-TCCAACCAGTCCCTACTCCACCCTCTA-3′	COI	Specific
Limno. COIF1	5′-TTTAGAGTTAGCACGTCCTGGTAGGTT-3′	COI	Specific

Continued

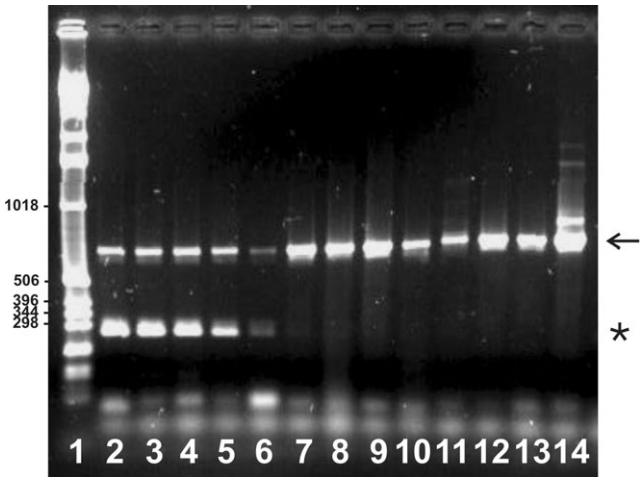

Figure 1. Gel illustrating the performance of the COI-specific primers to detect DNA from *Limnoperna fortunei*. Lane 1: 1 kb ladder. Lanes 2–6: results from sensitivity tests using increasingly smaller amounts of template DNA, i.e. 406, 40.6, . . . , 0.0406 ng. Lanes 7–14. Tests using *Corbicula fluminea*, *Modiolus brasiliensis*, a unionid bivalve, *Crassostrea gigas*, *Thais* sp., *Brachidontes* sp., *Colisella* sp., and *Perna perna*, respectively (other tested species not shown). The heavy product (←) is a fragment of 18S (universal), whereas the light product (*) is specific to *L. fortunei*. Collection sites are available from the authors upon request.

plankton samples taken in water reservoirs are being used to follow the expansion of the golden mussel and to optimize control efforts.

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