Testing a molecular protocol to monitor the presence of golden mussel larvae (*Limnoperna fortunei*) in plankton samples

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The golden mussel (Limnoperna fortunei, Mollusca: Mytilidae) is an emerging invasive species in freshwater environments in South America, causing extensive environmental and economic impacts. A molecular method to detect larvae of the golden mussel in plankton samples has been recently developed and holds promise for becoming an important way to monitor the expansion of golden mussel populations. In the present study, we conduct, for the first time, field tests of this method by comparing its performance with alternative sampling efforts (microscopy and manual search for adults). In addition, we test different modifications of the molecular method to deal with PCR inhibition in environmental samples. The results indicate that the molecular method is very efficient, being faster and more sensitive that microscopy methods. Therefore, the molecular method tested in the present study can represent an invaluable tool in large-scale monitoring efforts of the golden mussel throughout its introduced range.

INTRODUCTION

The golden mussel (*Limnoperna fortunei*, Mollusca: Mytilidae) has become an important invasive species in South American freshwater environments since it was first recorded in the Rio de la Plata estuary in 1991 (Pastorino *et al.*, 1993). It is currently found in Rio de la Plata, Rio Paraná, Rio Uruguay, and Rio Paraguay (Brugnoli *et al.*, 2005), as well as several lacustrine environments, such as the Lago Guaíba and the Lagoa dos Patos (Mansur *et al.*, 1999; Darrigran, 2001; Darrigran and Pastorino, 2003; Capitoli and Bemvenuti, 2004). *Limnoperna fortunei* is the only mytilid species living in freshwater environments. Contrary to other Neotropical freshwater mollusks, this species is characterized by external fertilization, a larval development similar to marine taxa (with planktonic veliger larvae), and the appearance of the bissus starting on the last larval stage. This mollusk is morphologically and functionally similar to the Holartic invasive mussel, *Dreissena polymorpha* (Pallas 1771) (Bivalvia, Dreissenidae) (Darrigran and Damborenea, 2006). As *D. polymorpha* in North America, the explosive population growth of the golden mussel has led to severe environmental and economic problems, particularly in the case of hydroelectric power plants and water treatment facilities (Darrigran, 2002; Boltovskoy *et al.*, in press).

Efficient management of the golden mussel in its introduced range requires the ability to predict sites most likely to experience colonization, permitting targeting of control measures. Such prediction can be aided by regularly

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monitoring the presence of its larvae in the plankton, particularly in sites where adults are still absent or cryptic. A PCR-based molecular method has been recently described for the detection of larvae of *L. fortunei* in plankton samples (Pie *et al.*, 2006). This method is based on the co-amplification of fragments of two genes. The first fragment is part of the cytochrome oxidase subunit 1 and is amplified with primers specific to *L. fortunei*. The second fragment serves as a positive control, amplifying a region of the 18S nuclear ribosomal gene using universal primers. If a given reaction lacks DNA from the golden mussel, the resulting PCR product should contain only the 18S fragment resulting from the amplification of DNA from any plankton species present in the sample.

Although this method is very efficient in discriminating and detecting golden mussel DNA, its performance using field-collected plankton samples has not been explicitly tested. Indeed, a major challenge for the detection of target DNA in environmental samples is that they almost invariably contain substances that are co-purified with template DNA and inhibit amplification. For instance, extremely small quantities of humic material (as small as 1 ng) have been shown to inhibit PCR (Menking *et al.*, 1999). Several methods have been developed to reverse inhibitory effects of these substances, particularly in soil (Straub *et al.*, 1995; Yeates *et al.*, 1998; Braid *et al.*, 2002; Van Dyke and McCarthy, 2002) and water samples (Gantzer *et al.*, 1997; Reynolds *et al.*, 1998). However, a comparison of different methods seems to indicate that a solution to the problem of PCR inhibition has to be sought in a case-by-case fashion.

In this study, we report on tests using three alternative methods to cope with PCR inhibition when applying the molecular marker to environmental samples, all of which involving the use of adjuvants in the PCR reaction: bovine serum albumine (BSA), dimethylsulfoxide (DMSO) and glycerol. Also, we tested the ability of the molecular marker to detect golden mussel larvae by applying it to environmental samples that had been spiked with known numbers of larvae. Finally, we test the performance of the molecular method for the detection of larvae of the golden mussel in field-collected environmental samples by cross-validation using the inspection of plankton samples using light microscopy and the prospection of adult mollusks.

METHOD

PCR inhibition tests

The performance of different adjuvants in neutralizing PCR inhibitors was tested using a plankton sample collected in the hydroelectric power plant of São Jorge, municipality of Ponta Grossa, PR in Southern Brazil, by filtering ~60 000 L of river water. In the laboratory, this sample was again filtered through a coarser mesh (150 μm in diameter) to remove small organisms (such as cyanobacteria), and then processed for DNA extraction with the EZ-DNA kit (Biossystems, Brazil) using manufacturer's instructions. Although such mesh diameter might have caused a loss of small ciliated, trocophorae and D-larvae of the golden mussel, the results indicate that the sensitivity of the method was not impaired (see below). Indeed, if a preliminary inspection of the plankton sample indicates a very low density of cyanobacteria, this step might be omitted altogether. The resulting solution was mixed with DNA extracted directly from tissue samples of the golden mussel. PCR reactions were conducted in 25 μl solutions with the following final concentrations: 1.5 mM of $MgCl_2$, 0.5 mM of dNTPs, 1X reaction buffer, 0.625 units of *Taq* platinum (Invitrogen), 4 mM of each specific (COI) primer, 0.8 mM of each universal (18S) primer, 0.47 ng/μL of plankton DNA and 3.38 ng/μL of golden mussel DNA. Previous tests showed that the DNA extract of golden mussel used in this study, when mixed with the DNA extract from the plankton sample, resulted in the complete inhibition of the reaction (not shown). The following adjuvant concentrations were tested: BSA at 0.2, 0.5 and 0.8 μg/μL, DMSO at 5% and 10% and glycerol at 5% and 10%. Primer sequences are listed in Table I. DNA from the golden mussel was included as positive control. Thermocycler settings included an initial denaturation period of 4 min at 94°C, followed by 32 cycles of denaturation (30 s at 94°C), annealing (30 s at 59°C) and extension (1 min at 70°C), ending in a final extension at 70°C for 3 min. PCR products were electrophorised on a 2% agarose gel, stained with ethidium bromide and visualized under UV light.

Experimental test using a known number of larvae

A sensitivity test of the molecular marker was performed with environmental samples spiked with known numbers of golden mussel larvae. To this end, 2000 m^3 plankton

Table I: List of primers used in the present study

Primer	Sequence	Gene
LIMNO.COIR1	5′-TCCAACCAGTCCCTACTCCACCCTCTA–3′	COI
LIMNO.COIF1	5′-TTTAGAGTTAGCACGTCCTGGTAGGTT–3′	COIn
7F	5′-GCCCTATCAACTTACGATGGTA–3′	18S
1100R	5′-GATCGTCTTCGAACCTCTG–3′	18S

samples were collected as described earlier at the Hydroelectrical Power Plants Jordão, Brazil (October, 2005) and Salto do Vau, Brazil (September, 2005), where the golden mussel is believed to be absent (see Table II for coordinates). The samples were processed as described earlier, and each was divided into three Eppendorf tubes to which one, five and zero (negative control) larvae were added. DNA extraction and PCR reactions were conducted as described earlier, except for the addition of BSA to a final concentration of 0.5 mg/mL. DNA from the muscle tissue of an adult golden mussel was included as positive control.

Testing the performance of the molecular marker on field samples

Samples were collected from a variety of locations (Table II) and submitted to the application of the molecular marker in association with the microscopic inspection of plankton samples of the same location, as well as the manual search for evidence of the presence of adults. Samples were obtained from several locations throughout the State of Paraná, Brazil, including 17 of the 18 hydroelectric power plants of the Companhia Paranaense de Energia Elétrica (COPEL) and from several locations in the State of Rio Grande do Sul (Table II). In each location, plankton samples were collected and processed as described earlier. These samples were either qualitative, in which a 40 μm mesh size net was dragged using a motor boat, or quantitative, in which a pump was used to filter 1000 L of water (200 L/min for 5 min). In either case, samples were fixed in 70% ethanol for analysis using optical microscopy (see Darrigran *et al.*, 2003 for on the used method) and finally preserved in absolute ethanol for molecular work. Adults were actively searched for attached to hard surfaces and aquatic plants in the margins of the sampled water bodies.

RESULTS AND DISCUSSION

A comparison of the performance of the different methods to prevent PCR inhibition in environmental samples is shown in Fig. 1. All three methods succeeded in reversing the inhibition that occurs in the absence of adjuvants. The addition of BSA was the most efficient in reversing the inhibition present in plankton samples, even under low concentrations (e.g. 0.2 μg/μL), generating products virtually indistinguishable from the positive control (Fig. 1). This result corroborates the usefulness of BSA in neutralizing inhibitors (Kreader, 1996). On the other hand, undesirable artifacts were present when using the other adjuvants. Instead of the single specific (COI) band, two bands of similar molecular weight were present in both DMSO and glycerol reactions. Moreover, the 18S (positive control) band was either very weak (glycerol 10% and DMSO 5% reactions) or failed to amplify altogether (DMSO 10%).

Sensitivity analyses using environmental samples spiked with a known number of larvae of the golden mussel in aliquots corresponding to 2000 m^3 of filtered water indicated that the molecular marker is sensitive enough to detect 1–5 larvae (Fig. 2). This result is consistent with previous tests using DNA from tissue samples of adult golden mussels (Pie *et al.*, 2006). These authors

Table II: Plankton samples tested for the presence of larvae of Limnoperna fortunei including both the molecular marker and cross-validation using light microscopy and the search for adult individuals

Location	Collection date	Coordinates	Marker	Microscopy	Adults
HP Foz do Areia (PR)	May 2005	51°40′23.1″S 25°59′23.6″W	–	–	–
HP São Jorge (PR)	April 2005	50°03′32.0″S 25°01′15.6″W	–	–	–
HP Figueira (PR)	July 2005	50°23′21.4″S 23°51′08.9″W	–	–	–
HP Pitangui (PR)	July 2005	50°05′13.5″S - 25°00′59.0″W	–	–	–
HP Rio dos Patos (PR)	August 2005	50°55′59.8″S 25°10′00.4″W	–	–	–
HP Melissa(PR)	September 2005	53°12′14.4″S 24°32′10.3″W	–	–	–
HP Cavernoso (PR)	September 2005	52°12′56.8″S 25°29′38.0″W	–	–	–
HP Chopim 1 (PR)	September 2005	52°45′00.0″S 25°59′0.23″W	–	–	–
HP Salto do Vau (PR)	September 2005	51°11′14.6″S 26°02′10.6″W	–	–	–
HP Jordão (PR)	October 2005	52°04′35.0″S 25°45′43.9″W	–	–	–
Canal São Gonçalo (RS)	February 2006	31°48.693′S 52°23.349′W	+	Abundant*	Few individuals
Lagoa Mirim (RS)	February 2006	32°30.298′S 52°34.995′W	+	-	4-5 dead individuals
Santa Isabela (RS)	February 2006	32°07.204′S 52°35.597 W	+	–	Many on the shore, on a boat
Santa Barbara (RS)	February 2006	31°48.693′S 52°23.349′W	+	Abundant*	Few individuals
Arroio Pelotas (RS)	February 2006	31°47.360′S 52°52.250′W	+	Scarce*	Few individuals
Bom Retiro (RS)	February 2006	29°36.465′S 51° 06.992W	+	0.58 larvae/L	Several on the walls of sluice

*, qualitative samples; –, negative; +, positive; HP, hydroeletrical power plant; PR, State of Paraná; RS, State of Rio Grande do Sul, Brazil. See text for details.

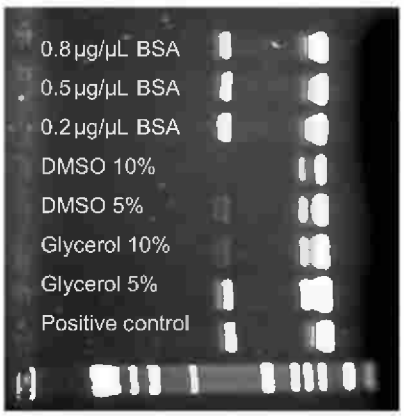

Fig. 1. Performance test of three alternative methods to minimize inhibition in PCRs from field-collected plankton samples. The positive control corresponds to the PCR of a sample containing only DNA from the golden mussel. The heavier band corresponds to the 18S fragment, whereas the lighter band indicates the presence of golden mussel DNA (COI fragment).

suggested that the method is capable of detecting as little as 0.041 ng of golden mussel DNA. The samples from Salto do Vau also failed to generate the positive control band, suggesting a problem during the application of the marker. Scrutiny of the samples indicated the use of an inadequate concentration of ethanol to preserve the samples, which might have lead to DNA degradation.

A list of sampled locations, the respective results from the application of the molecular marker, and the cross-validation using light microscopy and active search for adult golden mussel are shown in Table II. In the samples obtained from the State of Paraná, larvae of the golden mussel were still absent at the time of the collection. Other samples (not shown) failed to generate the positive control band, as described earlier. Later collections using good quality ethanol did not experience this problem.

No false negatives were detected in the studied samples. Interestingly, there were two instances in which light microscopy failed to detect golden mussel larvae, in contrast to a positive result of the molecular marker. Although this discrepancy could potentially indicate false-positive results, the presence of adults in those sites indicates that the molecular marker was more efficient than light microscopy in detecting the presence of golden mussel larvae in the plankton, in addition to providing results in a small fraction of the time necessary for microscopical analysis. For instance, the application of the molecular marker in the samples from the State of Rio Grande do Sul was accomplished around seven times faster than the microscopical analysis.

In conclusion, the results of the present study corroborate the efficiency of the molecular marker in the detection and monitoring of golden mussel larvae in its introduced range, and can represent an important tool for management efforts to control its populations.

Fig. 2. Sensitivity tests of the molecular marker using samples spiked with a known number of larvae of *Limnoperna fortunei*.

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