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RFLP pattern determination for the invasive bivalves *Limnoperna fortunei* (Dunker, 1857) and *Corbicula fluminea* (Muller, 1774)

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ABSTRACT

The golden mussel (*Limnoperna fortunei*) and *Corbicula fluminea* are considered well-established invasive species in the rivers of Brazil and South America. In addition to the environmental problems resulting from this invasion process, the economic issue, especially in hydroelectric dams, is very worrisome and has mobilized several types of studies on these invasive bivalves. The detection and identification of these organisms in their adult phase in the rivers is not a problem; however, the identification of bivalve larvae by usual morphological methods is difficult due to high similarity conserved in these stages. The use of PCR-RFLP has proven to be an efficient and agile molecular method that allowed the detection of different patterns in the agarose gel for the two bivalves tested. The gel pattern showed a 100 bp band for *L. fortunei* not detected for *C. fluminea*. Thus, it is possible to detect larvae of these species from water samples, which can be a powerful tool for environmental monitoring programs on aquatic invasive species.

Keywords: asia clam, golden mussel, larvae, molecular identification.

Determinação do padrão RFLP para os bivalves invasores *Limnoperna fortunei* (Dunker, 1857) e *Corbicula fluminea* (Muller, 1774)

RESUMO

O mexilhão dourado (*Limnoperna fortunei*) e *Corbicula fluminea* são espécies invasoras consideradas bem estabelecidas nos rios do Brasil e da América do Sul. Além dos problemas ambientais resultantes deste processo de invasão, a questão econômica, especialmente nas hidrelétricas, é muito preocupante e mobilizou vários tipos de estudos sobre esses bivalves. A

detecção e identificação desses organismos em sua fase adulta nos rios não são um problema, no entanto, a identificação de larvas de bivalves por métodos morfológicos habituais é difícil devido à similaridade extremamente conservada nestes estágios. O uso do PCR-RFLP se mostrou como um método molecular eficiente e ágil que permitiu a detecção de padrões diferentes no gel de agarose para os dois bivalves testados. O padrão do gel mostrou uma banda de 100 pb para *L. fortunei* que não foi detectado para *C. fluminea.* Desta forma com o uso de amostras de água é possível detectar os dois moluscos, o que pode ser uma ferramenta poderosa para programas de monitoramento ambiental em espécies aquáticas invasoras.

Palavras-chave: amêijoa asiática, identificação molecular, larvas, mexilhão dourado.

1. INTRODUCTION

Limnoperna fortunei (Dunker, 1857), or golden mussel, is a bivalve native to the Southeast of Asia. This specie was introduced in South America in the early 1990s when Mercosur's (the economic bloc from South America) business with South Korea and China was rising. The most-accepted hypothesis is that it entered along the coast of Argentina through ships' ballast water (Darrigran, 1995) with the first record in the Rio de la Plata/Argentina (Pastorino et al., 1993). Nowadays it is found in several regions of South America (Darrigran and Mansur 2006).

Another freshwater invasive bivalve is the *Corbicula fluminea* (Muller, 1774), or Asian clam, a Southeast Asia native species, which had its first American record in the USA by (Burch 1944 apud Mansur and Garces, 1988). Its arrival in South America occurred in the mid-60s and 70s, first recorded in the Rio de la Plata/Argentina estuary (Ituarte, 1981). Veitenheimer-Mendes (1981) recorded for the first time the presence of *C. fluminea* in Brazil, and since then several works showed its dispersion in the South (Mansur and Garces, 1988), Southeast (Avelar, 1999), Central-West (Callil and Mansur, 2002) and North (Beasley et al., 2003) regions of the country.

Among the various environmental problems in Brazil the presence of invasive species, which impact the environment, suppress native species and cause economic losses, are the most prominent. *Limnoperna fortunei* has caused several economic problems in hydroelectric plants and imbalance in the relationships between native fauna and flora (Brugnoli et al., 2005; Pejchar and Mooney, 2009) and *C. fluminea* is easily found in hydroelectric reservoirs throughout the country (Mansur et al., 2004a). Both are dioecious, with external fertilization and indirect development. The larvae consist of one of the main forms of dispersion of these organisms and reach a high adaptability success in the invaded environment due to their great density at the time of dispersion and the lack of specialized predators and parasites as adults (Darrigran and Mansur, 2006).

Bivalve larvae become a major problem when it comes to the identification of these organisms due to the difficulty of morphological distinction in microscopy analysis (Ludwig et al., 2014) at the most crucial moment for the management of invasive species during the early stages of colonization (Pie et al., 2017). Generally, optical identification techniques do not work with most bivalve larvae, since the specific characters appear only in the later stages of development or are visible only on a microscopic level (Garland and Zimmer, 2002).

Individuals have several diagnostic features only present during the adult phase; however, in some cases, morphological features, intuitively used for diagnosis, such as shell coloration, variations in shape and structure, muscle insertions scars, can be highly influenced by environmental factors (Pie et al., 2006a). The advent of molecular markers brings us powerful new tools to correctly identify individuals or groups that present a controversial morphological diagnosis at any stage of their development, being a notable instrument in systematics.

Molecular methods for detection of golden mussel were proposed using specific primers (Pie et al., 2006b), but they are restricted to binary responses (Pie et al., 2017), whereas technologies employing Real Time PCR (Endo et al., 2009; Pie et al., 2017) perform a quantification of larvae by indirect means, increasing the time and cost of execution. The same applies to the methodology used for the detection of *Corbicula* sp. (Ludwig et al., 2014) with the use of group-specific primers but which are not able to distinguish species.

The RFLP (Restriction Fragment Length Polymorphism) is a molecular method that has been successfully used to differentiate larvae of several mussel species such as the Pacific oyster, *Crassostrea gigas*, from the Portuguese oyster, *Crassostrea angulata* (Boudry et al., 1998). Pie et al. (2006a) also uses the same technique in his work to discriminate three species of oysters of the genus *Crassostrea*, grown on the Brazilian coast. Further, the RFLP assay was employed to determine the hemispheric origin of *Mytilus galloprovincialis* (Westfall et al., 2010).

The current work aims to use the RFLP technique to discriminate *Corbicula fluminea* and *Limnoperna fortunei*, the predominant mollusk invaders in South America (Ludwig et al., 2014), that can occur sympatrically causing ecological and economic damages. These species are morphologically distinct, but have in their larval stage a fundamental tool for identification of new invasions, complicated due to morphological differentiation.

2. MATERIALS AND METHODS

2.1. Samples collection

Adult specimens of each species were collected, placed in ethanol 70% and stored at -20°C. Table 1 shows the origin and collection points of the bivalves involved in this work. *Limnopena fortunei* was collected manually in the Rio Grande and *C. fluminea* by dragged sandbank in the mouth of Urucuia river.

Table 1. Collection points for each of the species used in this work.

Samples	Location	Coordinates
Corbicula fluminea	Foz do Rio Urucuia, São Romão, Minas Gerais - 15/01/2015	16°10'17.0" S, 45°32'20.0" W
Limnoperna fortunei (golden mussel)	Volta Grande hydroelectric dam, Miguelópolis, Minas Gerais	20°01'49" S, 48°12'59" W

All the collected bivalves were deposited in the collection of the Museum of Zoology of the Federal University of São Paulo.

2.2. DNA extraction

Following the material identification, separation of the foot and parts of the mantle was performed for DNA extraction. Scalpel blades were changed and tweezers were sterilized between specimens. For DNA extraction, the manufacturer's recommendations (Invitrogen®) were followed. The tissues were digested for 3,5 hours in a dry bath at 55°C and then subjected to the extraction protocol as instructed by the kit.

2.3. PCR

PCR was performed using universal primers for the gene sequence of the 18S ribosomal cytoplasmic RNA used by Ludwig et al. (2011). Primer Forward: 5'-GCCCTATCAACTTACGATGGTA-3' and primer Reverse: 5'-GATCGTCTTCGAACCTCTG-3'. The program used was: 94°C for 4 minutes; 32 cycles of

94°C for 30 seconds, 70ºC for 1,5 minutes, 70°C for 1 minute and extension at 70°C for 3 minutes extras. 100 ng of the extracted DNA, 0.05 U μL^{-1} of Platinum Taq DNA (ThermoFisher), 0.02 of µM primers were used for the reaction; 4 mM MgCl2; 0.04 mM of dNTP was added for a final reaction of 20 µL, with the volume adjusted with nuclease-free water. 1% agarose gel electrophoresis was performed.

2.4. Sequencing

The samples were sequenced by capillary electrophoresis in ABI3130 apparatus using POP7 polymer and BigDye v3.1, performed by Myleus Biotechnology (www.myleus.com). The comparison and confirmation of the sequences obtained were performed through the Basic Local Alignment Search (BLAST) program, available at https://blast.ncbi.nlm.nih.gov/Blast.cgi) and was deposited in GenBank (identified by codes: *Limnoperna fortunei*: MG674670 and *Corbicula fluminea*: MG674669) of the National Center for Biotechnology Information (NCBI). The sequences were aligned and analyzed in the BioEdit program (available at: http://www.mbio.ncsu.edu/bioedit/bioedit.html).

2.5. Enzyme digestion

The restriction enzyme *DdeI* used to generate the RFLP standard was chosen according to the standards generated by the NEBcutter software (available at: http://nc2.neb.com/NEBcutter2/). The restriction digestion of the PCR products occurred at 37°C for 2 hours in a total volume of 10 µl (7 µl of PCR product, 10 U μL^{-1} of DdeI [Promega] in 1X Buffer 10 mM Tris-HCl [PH 7.4], 50 mM NaCl, 0.1 mM EDTA, 1mM DTT, 0.5 mg mL^{-1} BSA, 0.01% Triton® X-100 and 50% glycerol [Promega]). The electrophoresis with the digested fragments was performed on a 1% agarose gel.

3. RESULTS AND DISCUSSION

The amplification of the 18S rRNA gene for invasive bivalves with about 700 base pairs (bp) is shown in Figure 1. The pattern of bands generated by the enzymatic digestion created distinct profiles among the species under study (Figure 2); for *L. fortunei* a band of ~100 bp is observed (Figure 2, lane 2) nonexistent for *C. fluminea* (Figure 2, lane 3). The observed pattern was the same for all replicates (*L. fortunei*, n = 14; *C. fluminea*, n = 11), which shows that the PCR-RFLP protocol described here can be applied for discrimination between invasive bivalve species. In addition, the results found for *L. fortunei* (n = 15) samples, which were collected at 2000 km to the North, and for *C. fluminea* (n = 13), collected at 780 km to the East of the original collection points, were identical, indicating that this method can be applied in other regions of Brazil.

The RFLP assay is quite robust and rapid for distinguishing larvae from *L. fortunei* and *C. fluminea* by performing only one detection test. The advantage of this method compared with the use of specific markers and Real Time PCR is to show a standard in agarose gel for the golden mussel, and in the absence of this we can confirm the presence of Asian clam. Due to the high genetic similarity between the other representatives of different species of *Corbicula* (Sousa et al., 2007), it would be hard to differentiate them among other species such as *C. largillierti* or *C. fluminlais*, for example (Ludwing et al., 2014). However, *C.fluminea* is a model organism for this study due to its vast dispersion (McMahon, 2000; Prezant and Chalermwat, 1984) and a massive expansion in sandy rivers (Hakenkamp and Palmer, 1999) like many spots in the São Francisco river basin, one of the most hydroelectric-potential rivers in Brazil. Standardizing a new form of fast and accurate detection of an invasive bivalve with the potential for ecological and economic damages such as *L. fortunei* is a tool of paramount importance in the work of state agencies or their contractors that manage hydroelectric power plants. This test

will be useful to monitor the introduction and possible dispersion of these bivalves in Brazil and South America, either by inlays in ship hulls, presence in ballast water or due to the aquaculture (Mansur et al., 2004b).

Considering the issue of biological invasions, it was suggested that work be done on prevention, an efficient alert system, a rapid response, and afterward work on invasion management (European Commission, 2013). Although easily distinguishable in adulthood, there is a difficulty, based on traditional morphological methods, to accurately differentiate the larvae of *L. fortunei* and *C. fluminea* (Ludwig et al., 2014). Thus, a rapid identification, whether of adult organism and/or its larvae, becomes necessary to identify newly invaded environments. The PCR-RFLP method is an assay that fulfills all these requirements. It is also a low-cost method which has the possibility of obtaining satisfactory results without the cost of sequencing the samples (Marescaux and Van Doninck, 2013).

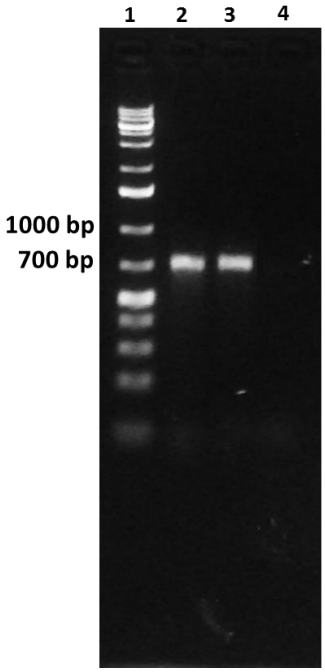

Figure 1. Amplification of 18S rRNA gene for invasive bivalves. Lane 1: 1 Kb Plus DNA Ladder (Thermo Scientific); lanes 2 and 3: 18S rRNA gene from *L. fortunei* and *C. fluminea*, respectively; lane 4: negative control of the reaction with the absence of DNA.

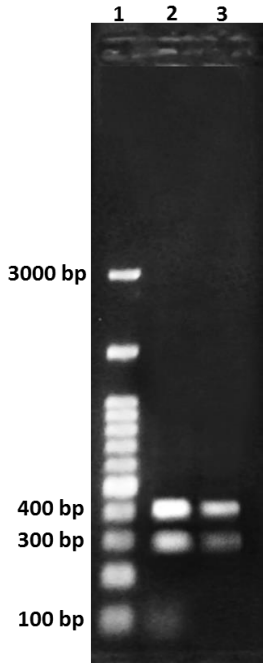

Figure 2. RFLP pattern for invasive bivalves. Lane 1: 100 bp Plus DNA Ladder RTU (Avati); lane 2: *L. fortunei* standard, evidencing the band of 100 bp, nonexistent for *C. fluminea*; lane 3: RFLP standard for *C. fluminea*.

4. CONCLUSIONS

This work showed that the PCR-RFLP can be used as a simple and rapid method to detect larvae of the golden mussel and species of Asian Clam, especially in samples with presence of larvae detected in morphological analyses. The clear RFLP pattern presented can help to confirm these invaders in watersheds, especially in environments with hydroelectric units. However, we recommend using the Ludwig et al. (2014) test if a 100 bp band is found in the PCR-RFLR tests and there is a need to confirm the presence of the Asian Clam.

New studies should also be conducted to assess the potential risks of these biological invasions (Pie et al., 2006a, Marescaux and Van Doninck, 2013), and reiterate that regardless of the choice, differents methods should be used for detection these mussels with the intention of getting more robust results in monitoring.

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