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Research Article

Looking for a needle in a haystack: molecular detection of larvae of invasive *Corbicula* clams

Sandra Ludwig*, Marcel K. Tschá, Raquel Patella, Annelise J. Oliveira and Walter A. Boeger

Laboratório de Ecologia Molecular e Parasitologia Evolutiva, Departamento de Zoologia, Universidade Federal do Paraná, C.P 19073, 81531-980, Curitiba, Paraná, Brazil

E-mail: sand.ludwig@gmail.com (SL), tschamarcel@gmail.com (MKT), raquelpatella@hotmail.com (RP), nelise8@gmail.com (AOJ), wboeger@gmail.com (WB)

**Corresponding author*

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Abstract

The invasive bivalves *Corbicula* spp. and *Limnoperna fortunei* predominate in South American rivers. They can be sympatric in distribution, and because their larval stages are morphologically similar, monitoring them in zooplankton using microscopy protocols is often inefficient, producing ambiguous results. We designed a pair of primers to amplify a fragment of the mtDNA cytochrome c oxidase subunit I of *Corbicula* species. A multiplex reaction, containing the specific primer pair and a pair of universal primers (to control for the quality of the DNA in the sample) was tested with regards to specificity and ability to detect *Corbicula* spp. larvae in plankton samples that also contain other species in different proportions. Our molecular protocol allows for fast and accurate detection of *Corbicula* spp. even when concentrations of these species are low in samples, which is useful when examining large volumes of ballast/piped water. Further, the protocol is valuable for the monitoring/prospecting of early stages of the life cycle of *Corbicula* spp. in watersheds that have been invaded, or which are considered at risk of invasion by these species.

Key words: mtDNA, Asian clam, molecular markers, zooplankton, prospecting, larvae

Introduction

Invasive species are often associated with loss of biodiversity (Rosa et al. 2011; Sousa et al. 2013; Pigneur et al. 2014), changes in native communities (Schlaepfer et al. 2005) and even accelerated extinction of native species (Clavero and Garcia-Berthou 2005). Additionally, some invasive species damage artificial structures and impact economic activities (Rosa et al. 2011). Successful invasive organisms, for instance the South American bivalves *Corbicula fluminea* (Müller, 1774) and *Limnoperna fortunei* (Dunker, 1857), often disperse efficiently using a combination of natural and human-mediated mechanisms (Cox 2004).

Corbicula clams and *L. fortunei* (the "golden mussel") were accidentally introduced to South America, most likely by ballast water (Darrigran and Pastorino 1993). These invasives often occur sympatrically (Darrigran 2002) and are still expanding their distribution in this continent (Oliveira et al. 2010). According to Pigneur et al. (2014), *Corbicula* spp. are particularly efficient invaders of river systems, reaching densities of up to several thousands of individuals per square metre in the Rio Paraná, Argentina. *Corbicula* spp. clams can reduce phytoplankton density and compete with native bivalve species of Mycetopodidae and Hyriidae (Santos et al. 2012). The golden mussel, on the other hand, has caused many problems for South American hydroelectric power plants by fouling in cooling ducts (Darrigran and Damborenea 2009; Belz et al. 2012). Management and control strategies need to be implemented for these species where they are present, and should include continuous evaluations of propagule pressure in new habitats (Darling and Blum 2007).

Active search for individuals is frequently used to monitor invasive bivalves. Adult specimens can be found in the substrate, and larval stages present in plankton are detected using optical

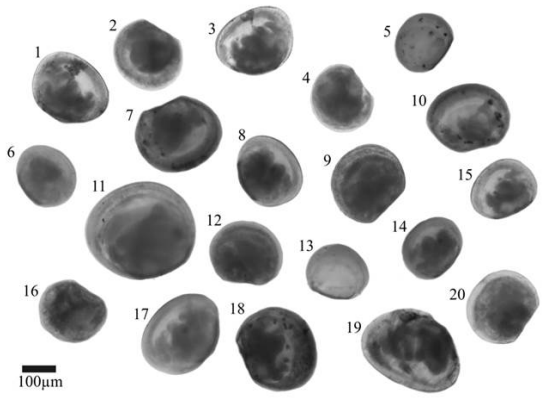

Figure 1. Larvae showing how similar *Corbicula* spp. (2, 4, 6, 7, 9, 10, 11, 12, 14, 16, 18, 20) and *L. fortunei* (1, 3, 5, 8, 13, 15, 19) are at these stages in their life cycle.

microscopy (Pestana et al. 2008; Lopes and Vieira 2012). However, zooplankton monitoring is often inefficient (Mansur et al. 2012a). According to Darrigran et al. (2009), this is in part because larvae of *Corbicula* clams and the golden mussel are very similar (Figure 1), which makes species determination under the microscope difficult, repetitive and tedious. One (untested) strategy that is often adopted by surveyors is to assume that all free-living bivalve larvae found in freshwater plankton samples are golden mussels. This strategy is based on the premise that all larval stages of native bivalves are exclusively parasitic (glochidia of Mycetopodidae and Hyriidae species; Mansur et al. 2012b; Gatlin et al. 2013) and/or that some species of *Corbicula* incubate their initial larval stages in the gills of their parents (Martins et al. 2006; Houki et al. 2011; Mansur et al. 2012b).

A molecular protocol for monitoring golden mussel larvae (Pie et al. 2006) has been widely used in hydroelectric power plants in Southern Brazil (Boeger et al. 2007). Combined with microscopic procedures, this protocol assists with larval identification and informs decision-makers regarding the need for management interventions such as the chemical control of larval settlements in the cooling system of turbines. However, this molecular protocol has failed to detect *L. fortunei* larvae in zooplankton samples in the past, even when large numbers of bivalve larvae were detected under the microscope. These results have prompted us to ask whether the early developmental stages present in these samples were in fact larvae of *Corbicula* spp., since no other freshwater species of bivalves in the freshwater environs of South America release larvae in the plankton.

We believe that markers are needed for species of *Corbicula* because the taxonomy of the genus is uncertain and species determination is difficult due to morphological plasticity/variability (Lee et al. 2005; Pigneur et al. 2011). As a consequence, the species composition of invasive *Corbicula* clams in South America and in other continents is largely questionable (Pfenninger et al. 2002; Lee et al. 2005; Hedtke et al. 2008; Pigneur et al. 2011). We therefore developed a molecular protocol for the detection of *Corbicula* species, similar to the one available for *L. fortunei* (Pie et al. 2006), with the following goals in mind: (i) to provide a tool to monitor the temporal and spatial availability of bivalve larvae; (ii) to facilitate identification of adults and larvae; (iii) to investigate whether free early larval stages of *Corbicula* (outside their parents' gills) are common in plankton samples, and (iv) to ascertain whether *Corbicula* spp. occur sympatrically with *L. fortunei* larvae in South America.

Materials and methods

Sampling and DNA extraction

Zooplankton samples and adult specimens of *Corbicula* spp. and *L. fortunei* were collected from reservoirs and Hydroelectric power stations (UHE) in southern Brazil (Table 1). Zooplankton samples were collected by filtering 4,000 L of water through a plankton net (64 µm mesh size), following Tschá et al. (2012). Two independent zooplankton samples were obtained from each collecting point and were preserved in 96% ethanol and taken to the laboratory. One zooplankton sample from each collecting point was processed under the dissecting scope and each identified bivalve larva was transferred to a microscope slide. Larval stage determination, based on Santos et al. (2005), was performed under a light microscope. Whole DNA extracts of zooplankton samples (from the second zooplankton sample per collection point) and DNA extracts from individual larvae were subjected to molecular protocols for the identification of *Corbicula* spp. and *L. fortunei*.

Total genomic DNA was extracted from the mantle tissue of adult *Corbicula* spp. (n=10 specimens) and one specimen of each *Crassostrea gigas* (Thunberg, 1793), *Modiolus brasiliensis* Chemnitz, 1795, *Thais* sp. (Röding, 1798), *Melanoides tuberculatus* (Müller, 1774), and from each bivalve larvae isolated from the zooplankton samples, using the EZ-DNA kit (Biosystems,

Table 1. Sampling sites for the collection of zooplankton and specimens of *Corbicula* spp. and *L. fortunei*. The use of specific molecular protocols (this work; Pie et al. 2006) determined the presence (+) or absence (-) of larvae of both species in each location.

Sites	Coordinates	Collection Date	Plankton Results		Larvae Results	
	Lat, S/Long,W		*L. fortunei*	*Corbicula* spp.	*L. fortunei*	*Corbicula* spp.
UHE1 Caxias	25°38'08" 53°20'43"	May 2011	+	-	24	-
Uruguay River	29°70'42" 56°33'28"	April 13th, 2011	-	+	-	19
UHE Jauru	15°12'51" 58°43'45"	October 2010	+	+	56	2
UHE Mauá	24°03'48" 50°42'05"	December 2011	-	+	-	27
Ibicuí River	29°35'50.82" 55°28'54.86"	April 14th, 2011	-	+	-	1
Das Antas River	29°05'16" 51°42'59"	April 14th, 2011	-	-	-	-
Jacuí River	30°03'48" 52°53'39"	April 15th, 2011	-	-	-	-
				Total	**80**	**49**

Brazil), following the manufacturer's instructions. The concentration of all DNA products was measured using a NanoDrop 3300 (Thermo Scientific).

Design of specific COI primers for Corbicula *spp*

A mtDNA fragment (700 bp approx.) from the cytochrome oxidase subunit I (COI) gene was amplified from the DNA of adult specimens of *Corbicula* spp. using a universal primer pair (LCO and HCO, Table 2). DNA was amplified in 25 µL reactions with 2–3 ng/µL of template DNA, 2 mM of $MgCl_2$, 0.4 mM of dNTPs, 1X buffer, 1.25 U of AmpliTaq DNA Polymerase and 0.5–1 mM of each primer. The following program protocol was used to obtain products: initial denaturation at 95°C for 5 min, followed by 35 cycles of 30s at 92°C, 30s at 48–51°C, 30s at 68°C, and final extension at 68°C, for 2 min. Amplified fragments were sequenced in laboratory, in both directions, using Applied Biosystems 3130 automatic sequencer and the same amplification primers. Sequences were assembled, edited and a consensus was generated using Geneious® 6.1.2 (Biomatters; Available at http://www.geneious.com/).

We compiled 25 COI sequences (600 bp approx. after trimming) derived from ten adults of *Corbicula* spp., as well as sequences from closely related species available on GenBank (Table 3), and aligned all these sequences based on the frequency of mismatches between them. Transversions and gaps were given more weight. Based on this alignment, unique regions were identified in the COI sequences of *Corbicula* spp., and a primer pair was designed to amplify 400 bp of their mtDNA (Table 2). Primer sequences were tested with GenBank's Basic Local Alignment Search Tool (BLASTn) (at https://www.ncbi.nlm.nih.gov/) to ensure that the designed primers would match only COI sequences of *Corbicula* spp.

Development of a Multiplex PCR assay

A multiplex PCR assay was developed using a pair of invertebrate universal primers that amplify an 800 bp fragment of nuclear 18S rDNA (Table 2) in addition to the specific COI marker that we developed for *Corbicula* spp. The universal 18S rDNA primer pair serves as positive control to account for variable DNA quality (Pie et al. 2006; King et al. 2009; Ludwig et al. 2011) and inhibition of the PCR of each individual sample. The Multiplex reaction was optimized by changing primer concentrations, DNA template concentration, and annealing temperature and time.

Specificity tests were conducted by testing the designed primers against samples of other mollusk species found in South America: *L. fortunei, C. gigas, M. brasiliensis*, *Thais* sp. and *M. tuberculatus* (some of these species were chosen also to test against species that could be found in ballast water). These tests ensured that the designed primers were specific to *Corbicula* spp., and that DNA from other species in a multiplex reaction would not result in cross-amplification.

Also, a sensitivity and specificity test was performed by adding the equivalent of the DNA content of one, two, four and sixteen bivalve larvae to total DNA aliquots derived from a zooplankton sample that did not contain *Corbicula* spp. (confirmed by the absence of the specific band in the application of the multiplex

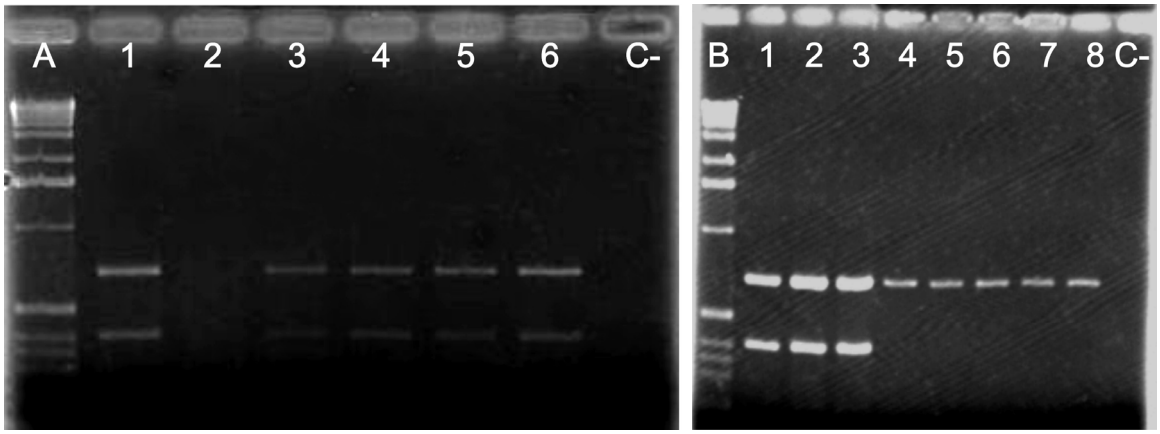

Figure 2. Performance of the *Corbicula* spp. markers. A) Larval detection test: (1) Extract of *Corbicula fluminea* adult; (2) Extract of plankton sample without *Corbicula* spp. DNA; (3) Plankton sample with DNA of one *Corbicula* larva; (4) Plankton sample with DNA of two *Corbicula* larvae; (5) Plankton sample with DNA of four *Corbicula* larvae; (6) Plankton sample with DNA of sixteen *Corbicula* larvae; (C-) Negative control. B) Specificity test: (1) Extract of *Corbicula fluminea* adult, (2) Extract of *Corbicula largillierti* adult, (3) Extract of *Corbicula* sp. adult, (4) Extract of *Limnoperna fortunei* adult, (5) Extract of *Crassostrea gigas* adult, (6) Extract of *Modiolus brasiliensis* adult, (7) Extract of *Thais* adult; (8) Extract of *Melanoides tuberculatus,* (C-) Negative control. For both tests, the upper band is the quality control markers and the lower band is the specific molecular markers of *Corbicula* spp.

Table 2. List of primers used in the development of the molecular detection protocol for *Corbicula* spp.

Primer	Sequence (5'- 3')	Gene	Primer type	Reference
LCO	GGTCAACAAATCATAAAGATATTGG	COI	Universal	Folmer et al. (1994)
HCO	TAAACTTCAGGGTGACCAAAAATCA			
CorbF2	GCTATTCCAGGGACTTTA	COI	Specific	This study
CorbR2	GCTCCAGGACGCATACAA			
7F	GCCCTATCAACTTACGATGGTA	18S	Universal	Modified from Telford (2000)
1100R	GATCGTCTTCGAACCTCTG			
Limno. COIR1	TCCAACCAGTCCCTACTCCACCCTCTA	COI	Specific	Pie et al. (2006)
Limno. COIF1	TTTAGAGTTAGCACGTCCTGGTAGGTT			

PCR designed herein). The DNA content of a single larva of *Corbicula* spp. was estimated from larvae of *L. fortunei* (Pie et al. 2006), since they have similar size (approximately 28.5 ng of DNA per larva). The equivalent volume of the simulated number of larvae was added to an extract of 50µL of the full genomic DNA of the zooplankton sample (500 ng/µL). This plankton sample was primarily composed of cyclopoid copepods, bivalve larvae, insect larvae, cladocerans, tardigrades, nauplii larvae and mites.

An additional test involved the use of environmental plankton samples obtained from distinct regions of Brazil (see Table 1). In order to identify each larva collected to the species level, and to evaluate the presence of the species in plankton samples, molecular markers specific for *L. fortunei* (e.g. Pie et al. 2006) were used in parallel with the molecular markers for *Corbicula* spp. developed in this study (Table 2).

Results

We designed a pair of primers to amplify a 400 bp fragment of the mitochondrial DNA COI gene of *Corbicula* spp. (Table 3). The optimized conditions for the Multiplex PCR assay are the following: initial denaturation of 5 min at 95°C, 35 cycles of 94°C for 30s, 44°C for 30s and 72°C for 40s, and final extension of 5 min at 72°C. The amplification reaction (25 µL) consisted of 3 mM of $MgCl_2$, 0.4 mM of dNTPs, 1X buffer, 2.5 U of AmpliTaq DNA Polymerase, and 4 mM of specific primers. PCR products were analyzed using electrophoresis in 1.5% agarose gel to compare the size of each amplified fragment with a marker of known size (Ladder 1Kb Invitrogen®). All sequences obtained by us from these fragments matched 100% with *Corbicula* spp. sequences in BLASTn (at https://www.ncbi.nlm.nih.gov/).

Table 3. Species list and their GenBank accession numbers used in the development of *Corbicula* spp. molecular markers.

Species	Location	GenBank number
C. fluminea	Korea	AF196269
C. fluminalis	Netherlands	AF269096-8
C. sandai	Japan	AF196273
C. fluminea	USA	U47647
C. japonica	Japan	AF196271
C. sp (form C)	Argentina	AF519512
C. sp (form A)	USA	AF519495-507
C. fluminea	France	AF269094
C. fluminea	Thailand	AF196270
Polymesoda caroliniana	USA	AF196276
Mya arenaria	USA	AF120668
Batissa violacea	Germany	DQ837726
Achatinella mustelina	USA	AY044338
Limnoperna fortunei	Brazil	DQ264395
Neocorbicula limosa	Argentina	AF196277

The specificity test revealed that the designed primers did not amplify the DNA of any other taxon tested (Figure 2A). The combined tests of sensitivity and specificity with plankton samples spiked with specific amounts of *Corbicula* spp. DNA, demonstrating that the protocol above is capable of detecting the DNA of a single larva when this DNA is pooled with DNA extracted from a plankton sample (Figure 2B).

As a whole, the quality of the DNA in the environmental plankton samples processed in this study was adequate (non-degraded), as indicated by the positive amplification of the 18S rDNA fragment (Figure 2A). After identifying the early bivalve larval stages in the zooplankton of Southern Brazilian rivers (Table 1) we separated 160 larvae for molecular identification, of which only 129 had adequate amounts of DNA for molecular analysis. After application of the molecular identification protocol on both species, 49 larvae were identified as *Corbicula* spp. and 80 as *L. fortunei* (Table 1). D-shaped larva (see Santos et al. 2005) was the most common stage found for both species. Larvae of *Corbicula* and *L. fortunei* were detected in sympatry by the Multiplex reaction applied to zooplankton samples and to individual larvae in one location, UHE Jauru (Table 1).

Discussion

Implementation of the specific molecular protocol to detect/identify *Corbicula* spp. larvae in environmental samples demonstrated that one or more species of this genus were present in the plankton of 4 out of 7 locations sampled. Since the current protocol cannot distinguish among *Corbicula* species, positive results may indicate the presence of one or more of the species recorded from Brazil (*C. fluminea, C. largillierti* and *C.* cf. *fluminalis*) (see Martins et al. 2004; Mansur et al. 2012a). Efforts are currently being made in our laboratory to improve this molecular protocol in order to differentiate among all species of *Corbicula* that occur in South America.

Our results contradict the notion that all species of *Corbicula* incubate in the demi-gills (e.g. Cataldo and Boltovskoy 2000; Martins et al. 2006) and suggest that their larval stages occur in sympatry with early larvae of *L. fortunei* (Table 1). Therefore, the widespread strategy currently used in South America for the microscopic detection and quantification of *L. fortunei* larvae, which assumes that all free-living bivalve larvae found in freshwater plankton samples are golden mussels, is inappropriate. While most freshwater *Corbicula* are hermaphrodites and ovoviviparous, with incubation in the maternal gill (e.g. Glaubrecht et al. 2006), some species do employ different reproduction modes, as reported by Byrne et al. (2000) for *Corbicula australis* (Deshayes, 1830). *Corbicula australis* is dioecious and incubates veliger to pediveliger larvae in the inner demibranchs. There are published records of *C. fluminea* being hermaphroditic, incubating juveniles in outer demibranchs and releasing planktonic veliger larvae (McMahon 2002). *Corbicula fluminalis* is not known to incubate larvae in its gills (e.g. Korniushin 2004).

Although the molecular detection protocol designed herein reveals only the presence/absence of *Corbicula* spp. larvae in plankton, this information can aid in studies on propagule pressure, which will allow for rapid effective management response and preparedness (e.g. Darling and Blum 2007). However, in order to increase the usefulness of the results derived from this method, the present protocol needs to be improved to allow quantification of larvae by real-time PCR, as proposed by Endo et al. (2009). Quantification of larvae of *L. fortunei* and *Corbicula* spp. is fundamental to guide methods of control, especially in the definition of dosages of anti-fouling and/or molluscicide products usually applied to semi-closed water systems such as cooling systems of hydroelectric power plants.

Early detection, which allows for rapid response, is crucial for integrated programs of management and control of invasive species (e.g. Molnar et al. 2008). However, early detection is often difficult when the invasive organism is small, inconspicuous and/or difficult to identify. Detecting invasive species during the first phases of an invasion, when they are still in low concentrations, is important for successful intervention. Therefore, our protocol represents an important tool to monitor and understand the biology and larval dispersal capacity of *Corbicula* species in continental waters. Similar monitoring has been applied systematically to *L. fortunei* since 2006 by technicians of the Instituto Lactec (http://www.institutoslactec.org.br/), COPEL (http://www.copel.com), ELEJOR (http://www.elejor.com.br/), Eletronorte (http://www.eln.gov.br), Tractebel (http://www.tractebelenergia.com.br/wps/portal/internet) and CEMIG (http://www.cemig.com.br) (unpublished information). We suggest that the molecular protocol to detect *Corbicula* spp. larvae is applied together with the protocol for the detection of *L. fortunei*. By doing so, technicians can decide on control measures to be adopted based on the propagules of the prevailing species detected.

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