A molecular method to detect and identify the native species of southwestern Atlantic *Crassostrea* (Mollusca: Ostreidae)

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ABSTRACT. Among oysters, species of *Crassostrea* (Sacco, 1897) are the most attractive to aquaculture. In Brazil, the genus is represented by *C. rhizophorae* (Guilding, 1828) and *C. brasiliana* (Lamarck, 1819). Because the maturation and breeding technology is not well developed for these species, aquaculturists need a reliable method to decide the correct time to place spat collectors in the field, and to identify both species, which are morphologically similar. In this study a specific Multiplex PCR protocol was developed, using one pair of universal primers from 18S rDNA as a positive control and a pair of specific primers for each target species. The sensitivity and specificity of the protocol was evaluated. It detected *C. rhizophorae* DNA in low concentrations, and *C. brasiliana* DNA in even lower concentrations. Further, the Multiplex PCR proved efficient in detecting DNA in concentrations equivalent to that of a single larva of each species, either separated or combined, when mixed with total DNA extract of a plankton sample representing 1000 L of filtered water. Field tests confirmed the applicability of the protocol, which holds the promise to become an important tool for aquaculture or conservation programs, allowing for the continuous monitoring of the life cycle of *C. brasiliana* and *C. rhizophorae*, by detecting the right periods of larval release and settlement.

KEY WORDS. Multiplex PCR; oysters; plankton; specific primers.

Oyster harvesting is probably one of the oldest forms of marine extractivism, possibly dating from the dawn of civilization. The development of modern oyster cultivation techniques has boosted production, which supplies the demands of an ever growing market (SPENCER 1990). Oyster cultivation has important socio-economic impacts for economically disadvantaged coastal populations. It provides additional income for traditional communities, which are losing their long-established sources of income due to the collapse of fisheries throughout the world (ERSE & BERNARDES 2008).

Among oysters, species of *Crassostrea* (Sacco, 1897) are the most attractive to aquaculture because they have large meat-to-shell ratio and rapid growth rate. Within *Crassostrea*, the most widely reared species is *Crassostrea gigas* (Thunberg, 1793), the Pacific oyster. It was intentionally introduced in various continents for commercial purposes (YU & LI 2007). According to DIJKEMA (1997), *C. gigas* would not be able to reproduce in many regions where it had been introduced (a prediction proved incorrect) for the Atlantic waters of South America, since naturalized banks of the Pacific oysters are now known from Ar-

gentina (CASTAÑOS *et al.* 2009) to the state of Santa Catarina, Brazil (MELO *et al.* 2010).

As an invasive species, *C. gigas* causes serious environmental problems, such as displacement of native species (PATIL *et al.* 2005), alteration of the structure of natural communities, and introduction of parasites and epibionts (GALLO-GARCIA *et al.* 2004, FAO 2006). In order to avoid new introductions of *C. gigas* in Brazil, state and federal laws (IBAMA 1998) presently control the translocation of juveniles and adults. Such control has led to an increased interest in the cultivation of native oyster species.

In the southwestern Atlantic coast, two native species of *Crassostrea* are known, *Crassostrea rhizophorae* (Guilding, 1828) and *Crassostrea brasiliana* (Lamarck, 1819) (PIE *et al.* 2006b, IGNACIO *et al.* 2000). While *C. rhizophorae* grows slowly, achieving a relatively small final shell size (at least in southern Brazil), *C. brasiliana* grows faster and achieves a larger size, holding some economic promise (CHRISTO & ABSHER 2006). Consequently, where *C. gigas* cultivation is forbidden, *C. brasiliana* cultivation is likely the best alternative for aquaculture.

However, spat production of *C. brasiliana* appears to be one of the major bottlenecks for the culture of the species in Brazil. The maturation and breeding technology is not well developed for the species, and specialized laboratories are still scarce (POLI & LITLLEPAGE 1998). Aquaculturists capture juveniles directly from nature, resulting in significant environmental impacts to natural beds. An alternative to this practice is to use spat collectors, as presently done in Brazil. Nevertheless, studies on the reproductive cycles and settlement patterns of native oyster species are lacking in the country, hindering collecting efforts.

Larval and seedling morphologies of *Crassostrea* species are highly conservative, hindering species determination. Consequently, ecological studies or efforts to determine the adequate time for placing collectors in the environment for aquaculture purposes often suffer. Differentiation of larval forms of *Crassostrea* species requires the use of Scanning Electron Microscopy (CHRISTO *et al.* 2010), a method that does not provide rapid and precise identification of species in continuous studies. Previous life cycle studies on local *Crassostrea* spp. likely reflect a mix of congeners or even species of other bivalve genera (e.g. CHRISTO 2006) and thus are of little practical interest.

Fortunately, molecular protocols are being developed to allow simultaneous detection and identification of one or more species of interest in different types of samples, including plankton samples (MORGAN & ROGERS 2001, ANIL *et al.* 2002, KOTTA *et al.* 2006, PIE *et al.* 2006a). Indeed, identification of adult species of *Crassostrea* by molecular markers is becoming a common procedure (WANG & GUO 2007, PIE *et al.* 2006b, PATIL *et al.* 2005). However, the basic limitations that exist today to implementing molecular methods are the financial costs of using them in monitoring studies. Molecular methodologies were developed by PIE *et al.* (2006a) for screening larvae of the golden mussel, *Limnoperna fortunei* (Dunker, 1857) in zooplankton samples. That method has been shown to be efficient for continuous monitoring in rivers and lakes of the state of Parana, Brazil (BOEGER *et al.* 2007, DARRIGRAN *et al.* 2009, TSCHÁ *et al.* 2009).

In this paper we describe a Multiplex PCR protocol based on a set of primers that can be used to simultaneously detect/identify, in plankton samples, larvae, seedlings and adults of *C. brasiliana* and *C. rhizophorae*. Our protocol may become an important tool for the continuous monitoring of the life cycle of *C. brasiliana* and *C. rhizophorae*, allowing researchers to detect temporal and spatial variations in larval availability in plankton and settlement. With this information in hand, aquaculturists may be able to select the best cultivation sites, and decide when to initiate spat collection, maximizing success in obtaining larvae and seedlings of each species of oysters. This protocol was successfully applied to plankton samples in the Southeast coast of Brazil to help coordinate the collection of naturally occurring spats for aquaculture.

MATERIAL AND METHODS

Adult oysters, spats and plankton samples were obtained from Guaratuba Bay, Paraná, Brazil (25°51.80'S48°38.20'W). Plankton samples (n = 34) were obtained bimonthly from January to June 2010, from three regions within the Guaratuba Bay (Fig. 1) by using a water pump to filter 1000 L of water through a plankton net (mesh size = 65 μm). To determine the volume of plankton samples, the water was pumped up into a container. The time was counted until the container volume was filled. This time is the equivalent to the total volume sampled in 1000 L of pumped water.

Spats (n = 246) were obtained from January to April, 2010, using collectors consisting of two sets of three-PVC plates. Collectors were placed in the Cabaraquara region of the Guaratuba Bay (Fig. 1), positioned at two depths, 30 cm and 1 m, and for thirty days. All samples were fixed in 92% ethanol in the field and then transported to the laboratory. DNA extraction and application of the multiplex PCR protocol proceeded as described above.
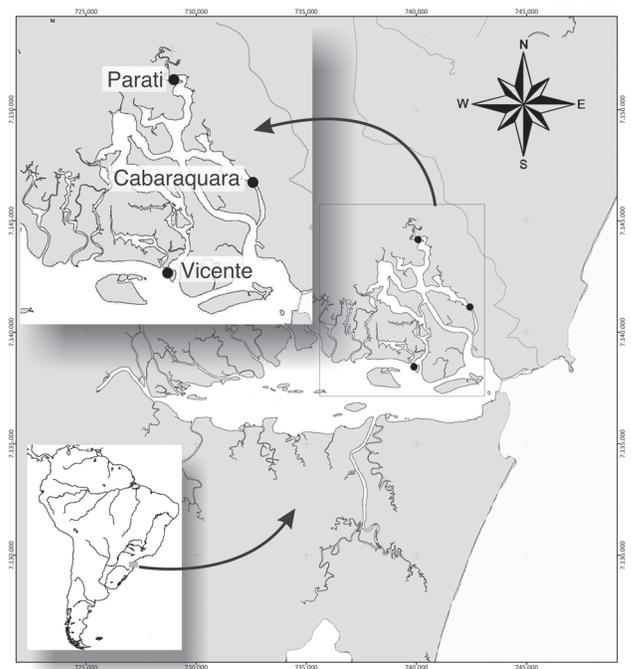
Figure 1. Location of the Guaratuba Bay and identification of collection sites for plankton and spat samples of native species of *Crassostrea*. Geographic coordinates are in UTM.

Pooled DNA from plankton samples was obtained in the following manner: a manual vacuum pump was used to filter the samples through paper filter. Organisms accumulated on

the filter were then used for DNA extraction. The total DNA was extracted using the EZ-DNA kit (Biosystems, Brazil), following the manufacturer's instructions. Adult oysters were identified using the protocol developed by Pie *et al.* (2006a).

A fragment of the mitochondrial 16S rDNA from tissues of adult oyster specimens was amplified with universal primers (16SAr and 16SBr, Tab. I) using the following PCR protocol: 4 min at 94°C/32 cycles of 94°C for 20s, 56°C for 40s and 72°C for 1min/72°C for 1min with 25 mL reactions with, 2.5U of AmpliTaq DNA Polymerase (Invitrogen®), 1X of PCR Buffer, 3mM of $MgCl_2$, 0.4mM of dNTPs, 0.4 µM of each primer, 2 ng/µL of extract of DNA and double-distilled water to complete total volume. PCR products were electrophoresed on 1.5% agarose gels and stained with ethidium bromide for band visualization and photo-documentation. Positive reactions were purified using the Minelute® (Qiagen, Germany) kit and cycle sequencing was carried out using the following final concentrations: 0.16µM of each primer, 0.25X reaction Buffer, 0.5 µL of BigDye v.3 (Applied Biosystems®), and 0.2-0.3 ng/µL of template DNA. Thermocycling conditions included an initial denaturation of 1 min at 96ºC, followed by 35 cycles of 10s at 96°C, 5s at 50°C, and 4min at 60°C. Products were purified using Sephadex™ G-50 medium (GE Healthcare Bio-Sciences AB) and both strands were sequenced on an ABI 3130 Automatic Sequencer. The sequences obtained were edited using the Staden package (STADEN *et al.* 1998). Representative sequences of each target species were deposited in the GenBank database under accession numbers HQ652329-HQ652331.

Table I. Primers used in PCR protocols.

Primers	Sequences (5'- 3')	Gene	Primer type
Ar	CGCCTGTTTATCAAAAACAT	16S	Universal
Br	CCGGTCTGAACTCAGATCACGT	16S	Universal
Crhiz_F	GCCCAGTGCGATATTAAGTC	16S	Specific
Crhiz_R	CGAACAGACCTACTCACT	16S	Specific
Cbras_F2	CACTGTCTCTTAGTTCTATG	16S	Specific
Cbras_R	AAGCCCTTTAGTTAATACGAG	16S	Specific
7F	GCCCTATCAACTTACGATGGTA	18S	Universal
1100R	GATCGTCTTCGAACCTCTG	18S	Universal

In order identify unique regions for designing specific primers, we compared the mitochondrial 16S rDNA sequences of *C. brasiliana* and *C. rhizophorae* with GenBank sequences of closely related mollusk species. Sequences from the following species, deposited on GenBank, were used: *C. gigas* (S66183.1), *Crassostrea* sp. (AY007426), *Crassostrea nippona* (Seki, 1934, AY510450.1), *Crassostrea rivularis* (Gould, 1861, EF122383), *Crassostrea belcheri* (Sowerby II, 1871, EU815968.1), *Saccostrea*

cuccullata (Born, 1778, DQ280032.1), *Ostrea edulis* (Linnaeus, 1758, AF458911.1) and *Saccostrea glomerata* (Gould, 1850, AY160756.1). All sequences were aligned using BioEdit (HALL 1999) and ClustaX l.2 (THOMPSON *et al.* 1997), and then adjusted visually. The identification of unique regions was based on the frequency of mismatches between the target species sequences and the sequences of the remaining species in the dataset, giving higher weight to transversions and gaps. We designed our primers using the software Primer Premier® (SINGH *et al.* 1998, Fig. 1), and conducted a preliminary test of primer specificity using BLAST (GenBank: www.ncbi.nlm.nih.gov/blast). If a given BLAST search resulted in close matching with sequences of other species, the primer pair in question was considered inadequate and new primer combinations were sought.

A Multiplex PCR was designed using one universal 18S rDNA primer pair (7F and 1100R, Tab. I) as positive control for the quality and presence of DNA in the sample, and primer pairs specific for the native species of *Crassostrea*. Thus, electrophoresis of a sample that does not contain either one of the target *Crassostrea* spp. sequences should result in a 18S rDNA stretch amplified by the universal primers. The absence of all fragments in a gel is interpreted as false negative which may have resulted from degraded DNA in the sample. The Multiplex reaction was optimized in an Eppendorf Mastercycler® Gradient in order to maximize sensitivity and specificity of the protocol, while attempting to attain electrophoresis bands of similar density for the same template concentration. The resulting electrophoretic gels (2% agarose) were subjected to densitometry with the software Gel-Pro Analyzer v4.0® (Media Cybernetics Inc., Silver Spring, MD), to improve the visualization of amplified fragments and separate the species identified by peaks corresponding to fragment size and position. A positive control, representing a sample of tissue of adult individuals of known identity and a negative control (lacking template DNA) were added to the protocol to confirm that the amplification was successful, and to control for possible contamination. The sensitivity of the optimized Multiplex PCR protocol was tested using extracts from adult tissue with 10-10.000 fold dilutions of an initial concentration of 2 ng/µL (0.2, 0.02, 0.002, 0.0002 ng/µL, respectively). A combined test for sensitivity and specificity was performed using the equivalent of the DNA content of a single bivalve larva (28.5 ng according to PIE *et al.* 2006a) added to the full extract of a zooplankton sample considered negative for larvae of both species of *Crassostrea* (1000 L of water filtered with 100 µm mesh size). Treatments were designed to simulate the absence, the presence of a single larva, and the simultaneous presence of one larva of each target species, submitted to the optimized multiplex PCR protocol.

Further, tests of the molecular protocol were conducted with plankton samples and spats described above. These samples were processed to evaluate the effectiveness of the proposed protocol in simulated field studies.

RESULTS

We designed and choose two sets of specific primer pairs to amplify solely a fragment of the mitochondrial 16S rDNA from *C. brasiliana* and *C. rhizophorae*, respectively (Tab. I). The Multiplex reaction was optimized with the following program: 4 min at 94°C/32 cycles of 94°C for 20s, 59°C for 44s and 72°C for 15s/72°C for 1min. in 10 µL reactions with 1U of TaqPlatinum (Invitrogen®), 1X of PCR Buffer, 3 mM of MgCl$_2$, 0.4 mM of dNTPs, 2 µM of each specific primer of *C. rhizophorae* and 1.2 µM of each specific primer of *C. brasiliana*, 0.1 µM of universal primer 7F, 0.1 µM of universal primer 1100R and, 2 ng/µL of extract of DNA and double-distilled water to complete total volume. After optimization of the reaction, the samples are processed more quickly. The initial reaction took two hours and ten minutes to complete. After optimization, the reaction time was reduced to one hour and thirty minutes. The sizes of the resulting fragments are as follows: 18S rDNA control: app. 700 bp long; *C. rhizophorae*: app. 450 bp long; *C. brasiliana*: app. 370 bp long.

The results obtained from the dilutions of DNA extracts from tissue samples of adult oyster demonstrate that the protocol detects low concentrations of oyster DNA (Fig. 2). The protocol detected the presence of the DNA of *C. rhizophorae* in

concentrations as low as 0.002 ng/¼L, and as low as 0.0002 ng/¼L, in the case of *C. brasiliana*.

Sensitivity and specificity of the primers proposed for the target species were also demonstrated by the test using extracts of zooplankton samples mixed with a DNA equivalent to that of a single larva (Fig. 3). Independent of the combination of each treatment, the method did successfully detect the target DNA of a single larva despite eventual competition with DNA of non-target organisms present in the samples. No nonspecific bands were detected in any treatment.

A total of 34 plankton samples were tested using the method proposed in this contribution. Larvae of *C. brasiliana* and *C. rhizophorae* were successfully detected every month of this experiment in Cabaraquara. In Parati, larvae of both species were detected only in January, whereas larvae of *C. brasiliana* represented the only species detected from April to June; no larvae of the target species were detected in the samples from February and March, 2010. In Vicente (sampled only after February, 2010), larvae of *C. brasiliana* and *C. rhizophorae* were detected only in April (Tab. II), both being absent in February, March, and June of 2010.

Finally, of the 246 spats collected, 38 (app. 16%) were identified as *C. brasiliana*; 58 oyster spats (app. 24%) were identified as *C. rhizophorae*; 82 were identified as a possible
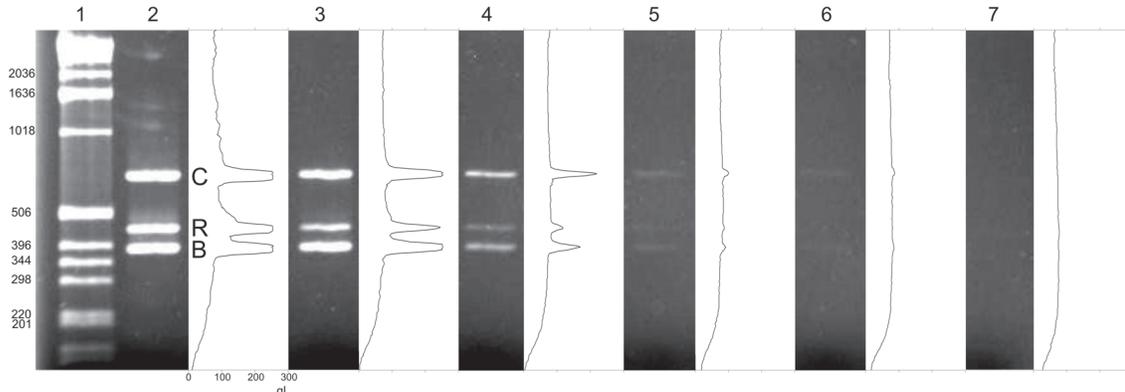
Figure 2. Agarose gel of the Multiplex PCR protocol for detection and identification of species of *Crassostrea*, native to the Brazilian coast. Each gel lane is paired with their respective optical density analysis. Lane 1: 1 kb ladder. Lanes 1-5 result from sensitivity tests using increasing smaller concentrations of template DNA, i.e. 2 ng/µL, 0.2, 0.02, 0.002, 0.0002. Lane 6: negative control. (C) quality control band (18S rDNA), (R) *C. rhizophorae*, (B) *C. brasiliana*, (GL) gross luminance.

Table II. Location of collections in Guaratuba Bay (Paraná, Brazil) and the results of the analysis of plankton samples with the protocol devised to detect larvae of species of native *Crassostrea* spp. (by month 2010). (B) Sample positive for *C. brasiliana*; (R) sample positive for *C. rhizophorae*; (–) negative for larvae of both species. No samples of Vicente were obtained in January 2010.

Collection Points	January	February	March	April	May	June
Cabaraquara (S25°49'59.8"W 048°34'41.6")	B/R	B/R	B/R	B/R	B/R	B/R
Parati (S25°48'02.5"W 048°36'25.0")	B/R	–	–	B	B	B
Vicente (S25°51.154"W 048°36.481")		–	–	B/R	–	–
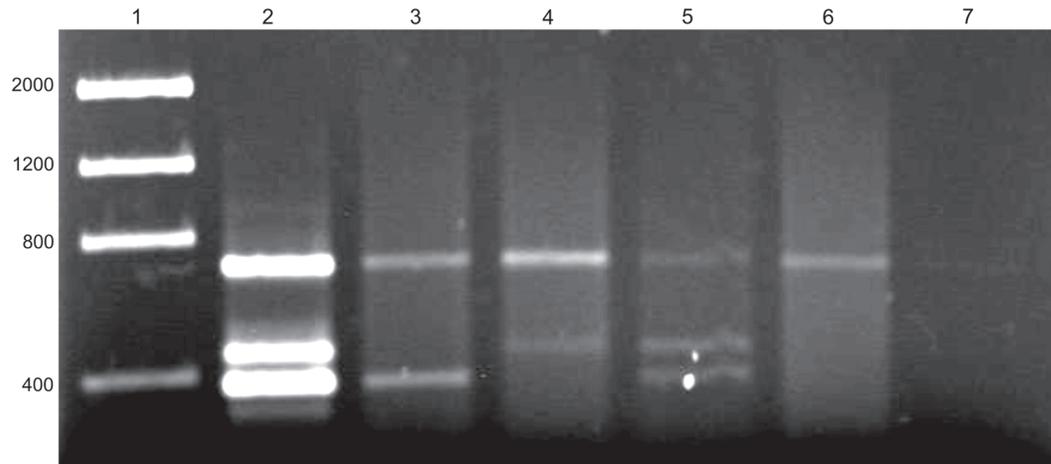
Figure 3. Sensitivity and specificity test of the Multiplex PCR protocol for detection and identification of species of *Crassostrea* native to the Brazilian coast. DNA-equivalent of a single larva (28.5 ng) was mixed with total extract of 1000-L-sample of plankton in different combinations: Lane 1. 1 kb Ladder. Lane 2: positive control lane (2 ng of adult specimens of each target species). Lane 3: DNA-equivalent of a single larva of *C. brasiliana* + total zooplankton DNA. Lane 4. DNA-equivalent of a single larva of *C. rhizophorae* + total plankton DNA Lane 5. DNA-equivalent of a single larva of *C. brasiliana* + *C. rhizophorae* + total plankton DNA. Lane 6. Total plankton DNA extract. Lane 7. Negative control.

Crassostrea sp. spat but could not be identified as either target species (specimens for which the control band was amplified but no specific band was visible); and 68 (28%) were not adequately preserved and did not yield accurate results (see discussion for more details). Both species were detected in collectors removed every month; unidentified spats were also present every month (Fig. 4). *Crassostrea rhizophorae* was relatively more frequent in January and February whereas *C. brasiliana* was more prevalent in February and March, and less prevalent in April. Unidentified spats were detected in greater numbers in April (these results were obtained after sequencing the 18S fragment). Samples with degraded DNA (detected by the absence of amplification of the universal/control 18S band) were more abundant in January and February but were significantly reduced in the last two months of the sampling period.

DISCUSSION

Laboratory and field tests support the efficiency of our Multiplex PCR protocol in detecting the target species even when DNA concentrations are low. Results from our simulations suggests that it is possible to detect at least one larva of each target species per 1000 L of filtered water in total plankton samples, without amplifying other species. If amplification of non-target species had happened, non-specific amplification bands would have been detected in our gels.

The results of our tests using environmental samples have demonstrated that our protocol permits the detection of larvae in plankton samples and the rapid identification of spats
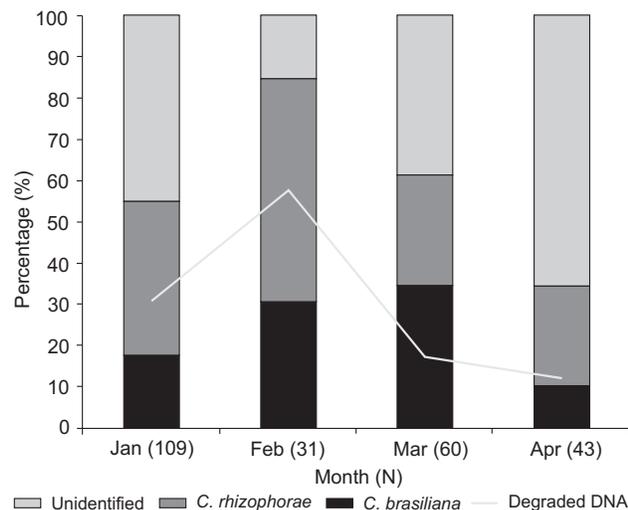
Figure 4. Percentage of *C. rhizophorae*, *C. brasiliana* and an unidentified species of oyster detected from January-April 2010 from collectors installed in the region of Cabaraquara (Guaratuba Bay, Brazil). The line graphic traces the percentage of specimens that could not be identified due to inadequate fixation and degraded DNA. The total number of specimens collected and processed each month is given in parenthesis following the month.

from collectors. Our results also suggest that the major obstacle to spat identification using PCR is poor preservation of fixed specimens, which results in loss of DNA integrity. Indeed, field

personnel failed to use enough ethanol when fixing spat samples in the months of January and February, resulting in significant loss of samples (Fig. 4). The fixation protocol was applied correctly on the spats removed from collectors in March and April, which resulted in a reduction in the percentage of degraded DNA (Fig. 4). Fixation was not a problem for plankton samples, as no false negative was detected.

Thus, the proposed protocol represents an important tool for continuous monitoring of the life cycle of *C. brasiliana* and *C. rhizophorae*, detecting temporal and spatial variations of larval availability in the plankton and settlement for each species. Although the sampling period of the field test was limited, it has provided evidence that there are differences in the spatial and temporal occurrence of larvae of the two targeted species of *Crassostrea*. Further, the identification and quantification of spats indicates distinct patterns of fluctuation in the settlement of *C. rhizophorae* and *C. brasiliana* in the same location (Cabaraquara, Fig. 1), which could represent important information on the differences in the life cycle of both species.

A rapid analysis of figure 4 strongly suggests that, in the region sampled, the peak of settlement of *C. rhizophorae* occurs between January and February, and that of *C. brasiliana*, between February and March. During this preliminary study we have also detected fluctuation in the availability of spats of other oyster species. These could represent either native species of other genera (not *Crassostrea*), or invasive species, as reported from other regions of the Southwestern Atlantic Coast (see Melo *et al.* 2010, Varela *et al.* 2007). Regardless of whether these organisms are native or not, their presence in the samples provides further evidence that the primers we designed are very specific, as they did not amplify the DNA of morphologically-similar spats (no cross-reaction).

If the life cycle and settlement of the two native *Crassostrea* species are asynchronous, as suggested by the results of the field test, we can predict the time individuals of each species settle by monitoring their larvae in the plankton. Based on such data, aquaculturists could ensure the identity of the majority of the seeds they collect by exposing the collectors in the environment during the right period and on the right site. The choice sites for installation of collectors appears to be another parameter to be considered to ensure the collection of oysters for aquaculture. From April to June, only larvae of *C. brasiliana*, the most adequate species for culture (at least in certain regions of the Southwestern Atlantic coast), were detected in plankton samples. The method developed in this study may also be used to complement morphological identification of spats and adult oysters, thus facilitating and maximizing success in selecting spats of the desired species by growers.

Even though we do not expect that false positive reactions will occur when using the proposed method, we recommend that researchers using it regularly sequence specific bands of the Multiplex PCR in order to confirm its precision.

Unfortunately, the present method is not capable to quantify larval density in plankton samples. However, our protocol could be easily adapted to allow an inference of the number of specimens of each target species though calibration with Real Time PCR, as developed by Endo *et al.* (2009) for *L. fortunei*. Subsampling of the plankton samples may also provide an alternative method to quantify the relative density of larvae of each species.

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