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Multiplex species-specific PCR identification of native and non-native oysters (*Crassostrea*) in Brazil: a useful tool for application in oyster culture and stock management

Mauro André Damasceno Melo · Adam Rick Bessa da Silva · Colin Robert Beasley · Claudia Helena Tagliaro

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Abstract In an effort to develop molecular tools for oyster identification, this study reveals the usefulness of a multiplex polymerase chain reaction (PCR) for the reliable, rapid and low-cost identification of the four oyster species found along the Brazilian coast: *Crassostrea gasar*, *Crassostrea rhizophorae*, *Crassostrea gigas* and *Crassostrea* sp. Canela originally found at Pará, Brazil. In order to perform a simultaneous identification of these species, we used a set of five primers developed and adapted from the cytochrome oxidase subunit I (COI) and internal transcribed spacer 1 (ITS1) fragments, respectively. Amplification was successful in all four species and PCR products were visualized in agarose gel. A single reaction was capable of distinguishing between the species: *C. gigas*, with two fragments (236 bp for COI and 718 bp for ITS1); *C. gasar*, with one fragment (718 bp for ITS1); *Crassostrea* sp., with one fragment (621 bp for ITS1) and *C. rhizophorae* with two fragments (377 bp for COI and 718 bp for ITS1). This molecular approach provides a simple and rapid identification of the oyster species from the Brazilian coast, thus increasing the efficient and quality of oyster culture programs by reducing the risk of wrong species identification.

Keywords Mangrove oyster · Management · Species identification · Stock conservation · Spat introduction

M. A. D. Melo (✉) · A. R. B. da Silva · C. H. Tagliaro
Laboratório de Conservação e Biologia Evolutiva, Instituto de Estudos Costeiros, Universidade Federal do Pará, Campus de Bragança, Alameda Leandro Ribeiro s/n, Bragança, Pará CEP 68600-000, Brazil
e-mail: mauro.melo@ifpa.edu.br; tagliaro@ufpa.br

M. A. D. Melo
Instituto Federal do Pará, Campus de Bragança, rua da Escola Agrícola s/n, Bragança, Pará CEP 68600-000, Brazil

C. R. Beasley
Laboratório de Moluscos, Instituto de Estudos Costeiros, Universidade Federal do Pará, Campus de Bragança, Alameda Leandro Ribeiro s/n, Bragança, Pará CEP 68600-000, Brazil

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Introduction

Oyster culture production worldwide is an increasingly important sector, reaching 4.5 million tonnes per year, and the genus *Crassostrea* is the fourth largest fisheries resource in the Western Atlantic (FAO 2010). Oyster production in South America (Atlantic coast) emerged as a result of the increased demand for shellfish, which led to the intentional and sometimes successful introduction of non-native species. In Brazil, attempts to enhance oyster production started in 1970s with the intentional introduction of the non-native Japanese oyster *Crassostrea gigas* in estuaries along the southeast coast, with imported oysters from Great Britain by the Cabo Frio Marine Research Institute in Rio de Janeiro state (Muniz et al. 1986; Littlepage and Poli 1999). In 1975, the São Paulo State Fisheries Institute brought *C. gigas* seed from Japan to Cananéia, São Paulo (Akaboshi 1979; Akaboshi et al. 1983), and in 1981, the Bahia Biology Institute imported *C. gigas* seed from Great Britain to implement oyster culture in northeastern Brazil (Ramos et al. 1986). The only successful attempt at oyster cultivation was that begun in 1987 in Santa Catarina state using Pacific oyster seed from Cabo Frio Marine Research Institute. Today, this state is considered Brazil's main producer of oyster (3,152 tonnes/year), representing 91 % of all oysters reared in the country (Oliveira Neto 2008).

For native species of Brazilian oysters, most of the efforts to cultivate remain restricted to subsistence by small family groups along the coast. Oyster seeds are obtained directly from the environment using artificial (plastic) collectors and grown using suspended lanterns and/or wooden tables in subtidal zones for up to 6 months or until market size is reached (at least 70 mm in length) (Galvão et al. 2009; Henriques et al.2010). Elsewhere, cultivation of both native oysters occurs in Cananéia, São Paulo state since 1970s (Wakamatsu 1973; Akaboshi and Pereira 1981; Akaboshi et al. 1983; Pereira et al. 1988, 2000, 2001a, b); Pereira and Chagas Soares 1996.

The use of morphological traits to correctly identify species of cupped oysters *Crassostrea* remains deficient due to the strong environmental influence on shell morphology during oyster development (Lam and Morton 2003). Singarajah (1980) considered *Crassostrea rhizophorae* Guilding, 1828 and *Crassostrea brasiliana* Lamarck, 1819 as synonymous. According to Absher (1989), two distinct patterns of growth and larval morphology are evidence of two species: *C. rhizophorae* Guilding, 1828 and *C. brasiliana* Lamarck, 1819. Nascimento (1991), based on morphological traits of the muscle scar, agreed with the existence of both species. Otherwise, Rios (1994) suggested that all Brazilian mangrove oysters belong to the species *C. rhizophorae*.

Some of the doubts about the correct identification of *Crassostrea* species from the Brazilian coast were classified when Lapègue et al. (2002) using RFLP-PCR and sequences of 16S rRNA confirmed the existence of two Brazilian native oysters (*C. rhizophorae* and *C. brasiliana*) and revealed the occurrence of a third mangrove oyster *Crassostrea gasar* Anderson, 1775 along the Atlantic coast of Africa and South America. Pie et al. (2006) deposited in the GenBank a *C. brasiliana* 16S rRNA sequence which was identical to the *C. gasar* fragment studied by Lapègue et al. (2002) suggesting that *C. brasiliana* and *C. gasar* are synonyms, the latter taking taxonomic precedence and more appropriate. Varela et al. (2007) and Melo et al. (2010b), using sequences of 16S rRNA and cytochrome oxidase c sub-unit I (COI), not only supported the existence of two native species but also identified the occurrence of another species provisionally named *Crassostrea* sp. Canela that is molecularly grouped with Indo-Pacific oysters. Melo et al. (2010a), using ribosomal 16S and the ribosomal second internal transcribed spacer (ITS2) sequences, reported the invasion of natural oyster beds by the Pacific oyster *C. gigas* in southern Brazil and

suggested that laboratory selection of seeds for growth rate and survival as the reason for the establishment of these species in the wild.

Given their economic importance, the correct taxonomic classification of oysters has important implications for oyster culture programs. The development of molecular techniques for rapid and reliable detection is required to prevent cultivation of the wrong species and to avoid accidental introduction of unwanted species. However, genetic approaches using DNA sequencing (Foighil et al. 1998; Huvet et al. 2000; Boudry et al. 2003; Wang et al. 2008; Melo et al. 2010b; Lazoski et al. 2011) is expensive and complex for routine identification. The use of restriction fragment length polymorphisms (RFLP) (Pie et al. 2006; Xia et al. 2009) requires, besides a polymerase chain reaction (PCR), additional steps and reagents to obtain the DNA digestion. The use of species-specific multiplex PCR for oyster identification is a more efficient and practical approach since a single reaction potentially distinguishes several species. Therefore, the aim of this study was to develop a suitable multiplex-PCR protocol to identify the four species of cupped oysters from natural beds along the Brazilian coast.

Materials and methods

We used the mitochondrial COI region to design a *C. rhizophorae* species-specific PCR inner primer (LCOINTCrh 5′-GCT ACC GGG GTC GTT ATT ACT C-3′) using the online program Primer 3 Plus (Untergasser et al.2007). For the identification of *C. rhizophorae* species-specific nucleotide positions and suitable annealing of the inner primer, 8 sequences of the *Crassostrea* genus were obtained from GenBank and aligned (Table 1). To measure any potential intraspecific variation, a total of 100 sequences of the species *C. rhizophorae* (obtained from Laboratório de Conservação e Biologia Evolutiva—LCBE) were aligned using the BioEdit v7.0.5.2 program (Hall 1999).

The COI region was also used to design the *C. gigas* species-specific PCR inner primer (LCOINTCgi 5′-CTT GCA ATT CTA AGC CTT CAC C-3′). A total of 23 sequences of *C. gigas* obtained from GenBank (AF280608, AY455664, AJ553909 to AJ553911, AY397685, AY397686, DQ417690 to DQ417696, DQ659367 to DQ659371, FJ717608, FJ743528, HM626169, HM626170) were aligned to check for intraspecific variation. COI sequences of thirteen *Crassostrea* species and one *Ostrea edulis*, obtained from GenBank (Table 2), were aligned using the BioEdit v7.0.5.2 program (Hall 1999). The main concern regarding the design of the *C. gigas* primer was the correct identification of the highly diagnostic sites that show no intraspecific variation and are distinct from other species of

Table 1 *Crassostrea rhizophorae* species-specific inner primers aligned with the COI region of 7 other species of oysters

Species	GenBank acession number	LCOINTCrh primer and aligned sequences
*Crassostrea rhizophorae**	HM003475	5'-GCTACCGGGGTCGTTATTACTC-3'
*Crassostrea gasar**	HM003499	TT....A..T..A....ACT.T
Crassostrea sp. Canela*	HM003525	T..T..A..A..TC.T.AT..T
*Crassostrea gigas**	AJ553910	TT.G..A.....TC.T.AT..T
Crassostrea angulata	AJ553908	TT.G..A.....TC.T.AT..T
Crassostrea ariakensis	FJ743527	TT.G..A..C..AC.T.AT..T
Crassostrea nippona	FJ743531	TT.G..A..T..AC.T.AT..T
Ostrea edulis	AF120651	T.G......T....AT.A...T

[a] Oyster species present along Brazilian coast

* Oyster species present along Brazilian coast.

Table 2 *Crassostrea gigas* species-specific inner primer aligned with the COI region of other species of oysters

Species	GenBank accession number	LCOINTCgi primer and aligned sequences
		380 401
*Crassostrea gigas**	AJ553910	CTTGCAATTCTAAGCCTTCACC
Crassostrea angulata	AJ553908	.....T.....
Crassostrea ariakensis	FJ743527	.....TT
Crassostrea nippona	FJ743531	.....T.....A..TT
Crassostrea sikamea	AF152568	.....T....TT.A....
Crassostrea belcheri	AY160755	.....T.G..A..A..T.
Crassostrea gryphoides	EU007489	..C..T...C.G..A..A....
Crassostrea iredalei	AY038078	.....T...T....G..G..TT
Crassostrea madrasensis	EU007462	.....T...T....G..G..TT
*Crassostrea rhizophorae**	HM003475	T....G...T....G..A..TT
Crassostrea sp Canela.*	HM003525	T.G.......G..GT.A...T
Crassostrea virginica	AF152566	T....C...T....GT.A...T
*Crassostrea gasar**	HM003499	T.G..C...T....GT.A...T
Ostrea edulis	AF120651	T.G.....T....AT.A...T

[a] Oyster species present along the Brazilian coast

* Oyster species present along the Brazilian coast.

oysters. A universal primer (HCO2198 5′-TAA ACT TCA GGG TGA CCA AAA AAT CA-3′) developed by Folmer et al. (1994) was included to anneal with both specific COI inner primers.

The second genetic marker used was the internal transcribed spacer 1 (ITS1) to discriminate between the species *C. gasar* and *Crassostrea* sp. Canela. This primer was originally designed by Pleyte et al. (1992) using the conserved 18S and 5.8S ribosomal DNA genes flanking the spacer region in salmonids (ITS1F 5′-AAA AAG CTT TTG TAC ACA CCG CCC GTC GC-3′ and ITS1R 5′-AGC TTG CTG CGT TCT TCA TCG A-3′).

A total of 158 samples—*C. gasar* ($n = 50$), *C. rhizophorae* ($n = 50$), *Crassostrea* sp. Canela ($n = 50$) and *C. gigas* ($n = 8$)—were collected along the Brazilian coast and tested for marker validation in the present study (Table 3). The adductor muscle of all oysters was preserved in 100 % ethanol and stored at −20 °C until DNA extraction. Genomic DNA was extracted using the phenol–chloroform protocol according to Sambrook et al. (1989). Samples of all these species were previously identified by means of mitochondrial COI and ITS1 sequences.

Polymerase chain reaction (PCR) for the COI primers (LCOINTCgi/LCOINTCrh/HCOC2198) and ITS1 primers (ITS1A and ITS1B) was performed in a 25 μL reaction volume. We tested different Mg++ concentrations and annealing temperatures in order to increase the primers' specificity. The optimized PCR was performed with 2.5 U of Taq Polymerase (Invitrogen®, Carlsbad, CA, USA), 100 ng of template DNA, 1.5 mM of $MgCl_2$, 2 mM of dNTPs, 1 × buffer solution, 0.3 μM of each COI primer and 0.24 μM of each ITS1 primer, followed by a cycling profile of 94 °C for 3 min, 40 cycles of 94 °C for 1 min, 60 °C for 1 min, 72 °C for 2 min, with an additional extension of 72 °C for 7 min for the last cycle. A 3 μL PCR product of each sample was electrophoresed during 80 min (50 V) in a 1.5 % agarose gel dyed in ethidium bromide and visualized under UV-light.

Results

The COI gene is highly variable among species of the genus *Crassostrea* and the sequence alignment of the four Brazilian oysters added with other species of this genus exhibited multiple unique nucleotide positions. Therefore, highly diagnostic sites containing a large number of unique nucleotides and showing no intraspecific variation were selected for

Table 3 Species and sample size used for the application of the species-specific multiplex reaction

Sample location[a]	Species[a]	Sampling location coordinates	Number of individuals
Canela Island, Bragança, Pará	*Crassostrea* sp.	00°47′02″S, 46°43′32.9″W	40
Nova Olinda, Augusto Corrêa, Pará	*Crassostrea* sp.	01°05′27.2″S, 46°28′28.6″W	10
São Cristovam, Sergipe	*C. gasar*	11°00′32,5′′S, 37°12′39,2′′W	25
Ilha das Fontes, São Francisco do Conde, Bahia	*C. rhizophorae*	12°40′0.0″S, 38°38′60″W	25
Búzios, Rio de Janeiro	*C. rhizophorae*	22°44′42.88″S, 41°52′57.30″W	25
Santos, São Paulo	*C. gasar*	23°52′18.55″S, 46°22′18.10″W	25
Florianópolis (SC)	*C. gigas* and *C. rhizophorae*	Oyster farm (matrices in captivity)	8

[a] Specimens were previously sequenced for COI and ITS1 sequences to identify the species

designing the inner primers of *C. rhizophorae* and *C. gigas*. No intraspecific variation at the inner primer annealing site was observed for the 100 analyzed sequences of *C. rhizophorae*. The inner primer, *LCOINTCrh*, presented 9 diagnostic nucleotide positions in comparison with *C. gasar*; 10 positions in comparison with *C. gigas* and 10 positions in comparison with *Crassostrea* sp. Canela. When analyzed with sequences of *C. angulata*, *C. nippona*, *C. ariakensis*, *C. gigas* and *O. edulis*, a minimum of 10 diagnostic nucleotide positions was observed.

The specific inner primer for *C. gigas* exhibited 7 diagnostic nucleotide positions in comparison with *C. rhizophorae*, 8 positions in comparison with *C. gasar* and seven positions in comparison with *Crassostrea* sp. Canela. The nucleotide divergence position between the inner primer annealing site and the four other species was 1 for *C. angulata*, 2 for *C. ariakensis*, 4 for *C. nippona* and 7 for *O. edulis*.

The ITS1 primer amplified a 718-bp fragment in all analyzed Brazilian cupped oysters, except for *Crassostrea* sp. Canela where a 621-bp fragment was clearly observed. All 50 specimens of this exotic species were amplified, and no evidence of length polymorphism was observed. Regarding the 718-bp fragment, all 108 collected samples including *C. gasar*, *C. rhizophorae* and *C. gigas* were analyzed and a similar absence of length polymorphism was observed.

The multiplex reaction was successful in all four species. The simultaneous combination of the COI species-specific inner primers and ITS1 primers in the PCR produced 2 fragments for *C. gigas* (718-bp length for ITS1 and 236-bp length for COI); 1 fragment for *Crassostrea* sp. Canela (621-bp length for ITS1); 1 fragment for *C. gasar* (718-bp length for ITS1) and 2 fragments for *C. rhizophorae* (718-bp length for ITS1 and 377-bp length for COI) (Fig. 1). All four Brazilian species can be positively identified in one single PCR. No evidence of cross-amplification was observed. In order to measure the reproducibility of the assays, all 158 samples were tested according to the protocol presented here and no evidence of distortion was observed, suggesting the reliability of the technique. One out of the eight samples obtained from the oyster farm located in Santa Catarina state was identified as *C. rhizophorae*, exhibiting two evident fragments equivalent to this native species (718 and 377 bp) and indicating the coexistence of *C. rhizophorae* and *C. gigas* in this locality.

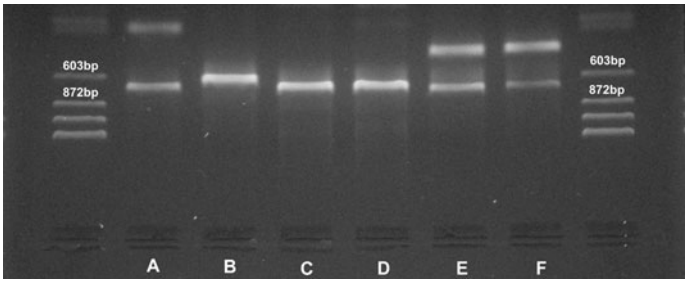

Fig. 1 Species-specific PCR multiplex of the four species of oyster (genus *Crassostrea*) from the Brazilian coast. *Lane 1* and *8*, *φ* X 174 DNA/*Hae*III Digest ladder; *lane A*, *C. gigas*; *lane B*, *Crassostrea* sp. Canela; *lanes C* and *D*, *C. gasar*; *lanes E* and *F*, *C. rhizophorae*

Discussion

Multiplex species-specific PCR is considered an effective and low-cost approach for the identification of oyster species (Hare et al. 2000; Larsen et al. 2005; Wang and Guo 2008). Two important points were considered during the development of the species-specific primers and multiplex reaction in the present study: the identification of diagnostic sites of single nucleotide polymorphisms (SNPs) and the possibility of genotyping these regions during the multiplex procedure. The specificity of the COI primers during amplification was a result of the high levels in interspecific variability of this gene, which is an important characteristic for the identification of several diagnostic SNPs for primer design. The use of ITS1 allowed us to distinguish between the exotic species *Crassostrea* sp. Canela and the native species *C. gasar*, an important achievement, especially for oyster cultivation programs on the northern coast where the co-existence of both species has already been reported (Varela et al. 2007; Melo et al. 2010a).

The name *Crassostrea* sp. Canela was first used by Varela et al. 2007 who classified this oyster as an Indo-Pacific species in terms of mitochondrial 16S RNA sequences. Melo et al. 2010b also supported these observations using mitochondrial sequences of COI. According to Gardunho et al. 2012, the status of exotic species increased when 16S RNA sequences of an unidentified *Crassostrea* species named *Crassostrea* sp. 2 (GenBank: HQ660984, HQ660988), from southern China (Liu et al. 2011), exhibited 99 % similarity to *Crassostrea* sp. Canela (GenBank: EF473278, EF473280 and EF473281). Galvão et al. 2012 compared COI sequences of oysters from Cananéia estuary (São Paulo, Brazil) with sequences of oysters from Canela Island (Pará, Brazil) (Varela et al. 2007) and the China Sea (Liu et al. 2011) and found no differences among the samples.

The exotic species *Crassostrea* sp. Canela is considered a problem for oyster farmers in this region. The miss-identification of these oysters in terms of morphological traits produces a considerable waste of effort and time during the cultivation process. Seeds of both species are collected simultaneously near natural beds and grown on tables. The non-native species does not reach commercial length incoming, an economic loss (approximately 30–40 % loss).

The use of this molecular identification technique in oyster cultivation programs may result in improvements in the sector. When performed, the multiplex species-specific PCR can simultaneously identify the four species of *Crassostrea* from the Brazilian coast. As these species can be distinguished through a simple, reliable, rapid and low-cost procedure, either small groups of oyster farmers or governmental monitoring agencies may use this

molecular approach as a routine procedure. The reported existence of the Japanese oyster in natural oyster beds along the south coast (Melo et al. 2010b) and the Indo-Pacific species *Crassostrea* sp. Canela from the northern coast of Brazil (Varela et al. 2007, Melo et al. 2010a) justify the need for a frequent evaluation of these stocks.

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