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SHORT COMMUNICATION

A simple PCR-RFLP method for the discrimination of native and introduced oyster species (*Crassostrea brasiliana, C. rhizophorae* and *C. gigas*; Bivalvia: Ostreidae) cultured in Southern Brazil

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Brazilian oyster production has expanded considerably over the past decade, increasing from 55 tonnes in 1995 to 2196 tonnes in 2005 (FAO 2005). In addition to the native oyster species, *Crassostrea brasiliana* (Lamarck 1819) and *C. rhizophorae* (Guilding 1828), the Pacific oyster, *C. gigas* (Thunberg 1793), are becoming increasingly prevalent in oyster farms throughout Southern Brazil. Moreover, the Pacific oyster has already spread into natural habitats in South America, raising concerns regarding the potentially deleterious environmental impacts of this species in its introduced range (e.g. Escapa, Isacch, Daleo, Alberti, Iribarne, Borges, Dos Santos, Gagliardini & Lasta 2004).

Monitoring the presence of various oyster species in natural habitats is not a straightforward task because of the difficulty in correctly discriminating different species. Morphological traits such as shell colour, form, structure and muscle scar are highly influenced by environmental conditions (Ignacio, Absher, Lazoski & Solé-Cava 2000), a problem that has for long plagued oyster systematics in general (Gunter 1951). Fortunately, the advent of molecular markers is bringing new and powerful tools to address a variety of issues in oyster studies, from settling systematics issues (e.g. Jozefowicz & O'Foighil 1998, Ignacio *et al.* 2000) to investigating species distributions (Lapègue, Boutet, Leitão, Heurtebise, Garcia, Thiriot-Quiévreux & Boudry 2002). In particular, RFLP methods have already been successfully used to discriminate the Pacific oyster from the Portuguese oyster, *C. angulata* (Boudry, Heurtebise, Collet, Cornette & Gerard 1998). In this study, we describe a simple PCR-RFLP that can be used to discriminate the oyster species cultivated in the Brazilian coast (*C. brasiliana*, *C. rhizophorae* and *C. gigas*).

Oyster samples were obtained in August of 2005 in the Ilha das Peças (25°27′26″S and 48°20′09″W) and in the Baía de Guaratuba (25°49′52″S and 48°34′43″W), State of Paraná, Southern Brazil. Voucher specimens were deposited in the Museu de História Natural da Pontifícia Universidade Católica do Paraná, Brazil. Genomic DNA was extracted from fresh specimens using the EZ-DNA kit (Biosystems, Curitiba, Brazil) according to the manufacturer's instructions. Amplification of a 530 bp region of the 16S rDNA gene was carried out using the primers 16S.AR (5′-CGCCTGTTTATCAAAAACAT-3′) and 16S.BR (5′-CCGGTCTGAACTCAGATCACGT-3′) (Palumbi, Martin & Romano 1991). Amplifications were performed in 25 μL reaction volumes with 2.5 U of Taq Polymerase (Invitrogen®, Carlsbad, CA, USA), 0.08 ng μL^{-1} of template DNA, 1.5 mM of $MgCl_2$, 0.5 mM of dNTPs, 1 × buffer solution and 2 μM of each primer, and

the following cycling settings: 4 min at 94 °C, followed by 32 cycles of 94 °C for 30 s, 56 °C for 40 s and 1 min at 72 °C and a final 1 min step at 72 °C. The restriction digestion of the PCR products was performed at 37 °C in a total volume of 10 μL using 7 μL of DNA template and 0.5 mL of *Hae*III restriction enzyme (Invitrogen®) in 1 × NEBuffer 2 solution (50 mM NaCl, 10 mM Tris-HCl, 10 mM $MgCl_2$, 1 mM dithiothreitol, pH 7.9; Invitrogen®). Digestion fragments were electrophoresed in a 3% agarose gel, dyed in ethidium bromide and visualized under UV-light.

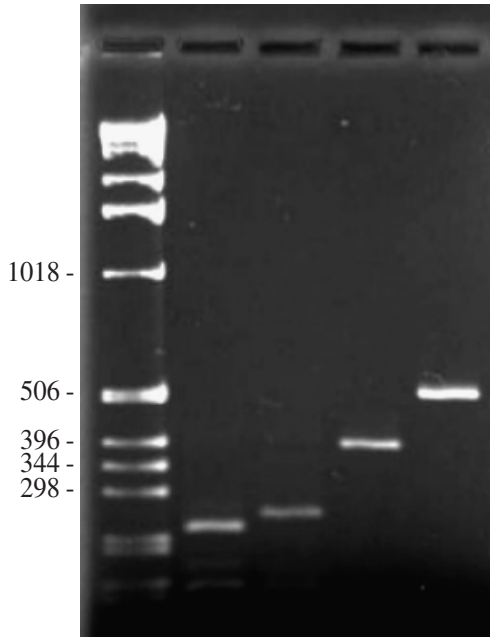

Figure 1 PCR-RFLP of a fragment of the 16S mtDNA gene of the three studied species. lane 1, 100 kb ladder; lane 2, *Crassostrea brasiliana*; lane 3, *C. rhizophorae*; lane 4, *C. gigas*; lane 5, undigested 16S fragment.

The bands resulting from the digestion of the 16S fragment of all three studied species showed very distinct profiles (Fig. 1). The same patterns were consistent across all replicates (*C. brasiliana*, $N = 15$ individuals; *C. rhizophorae*, $N = 12$ individuals and *C. gigas*, $N = 26$ individuals), indicating that the PCR-RFLP protocol described here can be successfully used to discriminate these three oyster species. Additional tests using *C. brasiliana* collected approximately 250 km to the South and 2000 km to the North of the original collections ($N = 20$ and 29 individuals respectively) showed identical results, indicating that the method can be applied in other regions of the Brazilian coast as well. Finally, further tests showed that extracts from oyster seeds were equally as effective in generating clear bands as tissues from adult individuals. The exact sizes of the RFLP bands were obtained by sequencing the 16S fragment for *C. brasiliana* and *C. rhizophorae* (GenBank accession numbers DQ839413 and DQ839415) and locating the digestion sites along the respective sequences. The expected size for the *C. gigas* samples was inferred from six sequences already available in GenBank. The results are consistent with the RFLP gels (Fig. 2). Although the smaller fragments cannot be reliably detected in 3% agarose gels, the largest fragment of each species (235 bp in *C. brasiliana*, 261 bp in *C. rhizophorae* and 395 bp in *C. gigas*) are sufficient for their consistent identification.

An important application of the protocol described in the present study would be to carry out large-scale monitoring programmes in natural habitats to understand the spatial and temporal extent of the Pacific

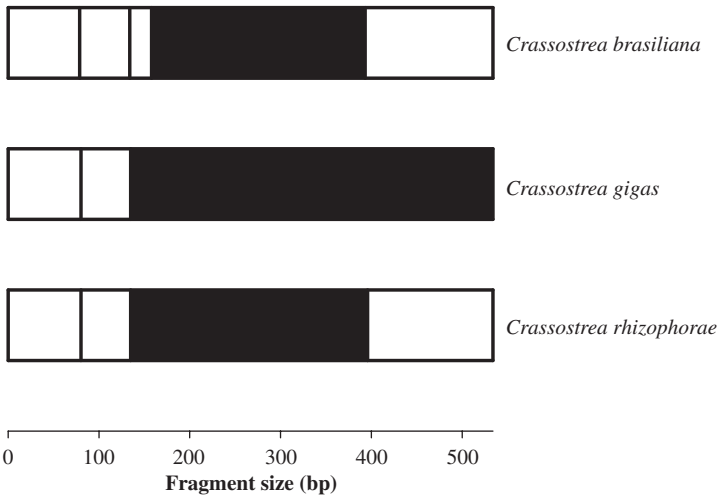

Figure 2 Diagram showing the digestion sites of a fragment of the 16S mtDNA gene of the three studied *Crassostrea* species using the enzyme *Hae*III. Sequences of *C. gigas* were obtained from GenBank (accession numbers: AF280611, AY632550, AJ553905, AJ553904, AJ553903, S66183). The large resulting fragments (black) are used for species identification.

oyster distribution in natural sites throughout the Brazilian coast and its interaction with native species. Although this species is only known to be cultivated in the Southern Brazilian states, several studies indicate that the temperature tolerance of the Pacific oyster may exceed 30 °C (e.g. Le Gall & Raillard 1988; Bougrier, Geairon, Deslous-Paoli, Bacher & Jonquières 1995), suggesting that it could indeed invade higher latitudes. In addition, the identification of oyster seeds using molecular methods can allow for a genetic certification of the identity of commercialized seeds.

By purchasing genetically certified oyster seeds, producers would ensure that their cultivated oysters are not jeopardized by growing a species that is not adapted to local environmental conditions. Finally, this method can help environmental agencies to monitor the presence of the Pacific oyster in different regions, thus preventing its indiscriminate cultivation and dissemination.

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