



## Molecular identification, phylogeny and geographic distribution of Brazilian mangrove oysters (*Crassostrea*)

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### Abstract

Oysters (Ostreidae) manifest a high degree of phenotypic plasticity, whereby morphology is of limited value for species identification and taxonomy. By using molecular data, the aim was to genetically characterize the species of *Crassostrea* occurring along the Brazilian coast, and phylogenetically relate these to other *Crassostrea* from different parts of the world. Sequencing of the partial cytochrome oxidase c subunit I gene (COI), revealed a total of three species of *Crassostrea* at 16 locations along the Brazilian coast. *C. gasar* was found from Curuçá (Pará state) to Santos (São Paulo state), and *C. rhizophorae* from Fortim (Ceará state) to Florianópolis (Santa Catarina state), although small individuals of the latter species were also found at Ajuruteua beach (municipality of Bragança, Pará state). An unidentified *Crassostrea* species was found only on Canela Island, Bragança. *Crassostrea gasar* and *C. rhizophorae* grouped with *C. virginica*, thereby forming a monophyletic Atlantic group, whereas *Crassostrea* sp. from Canela Island was shown to be more similar to Indo-Pacific oysters, and either arrived in the Atlantic Ocean before the convergence of the Isthmus of Panama or was accidentally brought to Brazil by ship.

**Key words:** genetic identification, oysters, biogeography, COI gene, Ostreidae.

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### Introduction

Morphological identification of cupped oysters *Crassostrea* to the species level is difficult, due to the intense environmental influence on shell development (Lam and Morton, 2003). Gunter (1950) concluded that, as to the shape of the shell, the oyster is one of the most variable bivalves in the world. Thus, the number of native cupped oyster species from the South American coast remains uncertain. *Crassostrea brasiliiana* (Lamarck, 1819) and *Crassostrea rhizophorae* (Guilding, 1828) were initially described from South American Atlantic mangroves (Nascimento, 1991). Great differences in growth rates and larval

morphology also lend support to the classification of Brazilian oysters into these two species (Absher, T M. PhD Thesis. Instituto Oceanográfico, USP, 1989). On the other hand, Singarajah (1980) proposed that *C. brasiliiana* and *C. rhizophorae* are synonymous, and so, based on morphological and physiological characteristics, described a new species, *Crassostrea paraibanensis*, from the Paraíba river estuary (Paraíba state, Brazil). Furthermore, he suggested the existence of another, as yet, unidentified *Crassostrea* species from the Tijuca lagoon (Rio de Janeiro). Rios (1994) considered all the Brazilian *Crassostrea* morphotypes synonymous with *Crassostrea rhizophorae*.

The only species that was deliberately introduced into Brazilian waters is the Pacific or Japanese oyster, *Crassostrea gigas*, which is cultivated in the cooler, southern waters (Littlepage and Poli, 1999). However, there have been no reports of attempts to cultivate Indo-Pacific oysters in northern Brazil. Along the northern coast (Pará state),

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some oyster farms have used native oyster larvae from different parts of northeastern Brazil for ongrowing (personal observation).

Biochemical and molecular genetic evidence (Ignacio *et al.*, 2000; Melo *et al.*, 2010) support the existence of two native species of *Crassostrea*, identified as *C. brasiliiana* and *C. rhizophorae*. On the other hand, Lapègue *et al.* (2002), on using both molecular evidence (rRNA 16S sequences and RFLP haplotypes) and karyological analysis, discovered the presence of two species from the South American coast, namely *Crassostrea gasar* (Adanson, 1757) and *C. rhizophorae*. Varela *et al.* (2007), based on 16S sequences, agreed with the latter authors and mentioned the presence of a third *Crassostrea* species in north Brazil, that was more closely related to Indo-Pacific oysters.

The inconsistency concerning *C. gasar* and *C. brasiliiana* was clarified when a *C. brasiliiana* rRNA 16S sequence deposited in the GenBank (DQ839413) by Pie *et al.* (2006) was compared with that of *C. gasar* (AJ312937) studied by Lapègue *et al.* (2002). Both sequences are identical, an indication that they belong to the same species. Furthermore, Nascimento (1991) mentions the presence of *C. brasiliiana* in Cananéia (São Paulo state, Brazil) and Ignacio *et al.* (2000) sampled *C. brasiliiana* from Paranaguá Bay (Paraná state, Brazil). Moreover, Lapègue *et al.* (2002) obtained samples of *C. gasar* at these same localities. Thus, to date molecular and biochemical evidence confirms the presence of two common species along the Brazilian coast: *C. rhizophorae* and *C. gasar* (*C. brasiliiana*). Based on this evidence, we will refer to *C. brasiliiana* by its former name, *C. gasar*.

Molecular and biochemical research, aimed at characterizing oyster species, has intensified worldwide. Ó Foighil *et al.* (1998) and Boudry *et al.* (1998) showed that *Crassostrea angulata*, derived from an Asian population of oysters, had only recently been introduced into Europe. Huvet *et al.* (2000) report evidence of the presence of two stocks of introduced Asian oysters in Europe: *Crassostrea gigas* and *Crassostrea angulata*. Nevertheless, Reece *et al.* (2008) were unable to distinguish between the two, when using COI parsimony analysis. Based on allozyme data, Day *et al.* (2000), besides noting a high degree of homogeneity among cultured populations of *Crassostrea* from Thailand, found that almost all the *Crassostrea belcheri* cultures examined had been contaminated with *Crassostrea lugubris* (= *Crassostrea iredalei*). The 16S rRNA and COI sequences of *Crassostrea* from the Pearl River delta, Hong Kong, were found to be distinct from those of other *Crassostrea* (Lam and Morton, 2003; Boudry *et al.*, 2003), whereat, by using morphological and molecular data, a new species, *C. hongkongensis*, was described (Lam and Morton, 2003).

*Crassostrea* species and their distribution in Brazil are poorly known. Based on COI sequences, our aim was to

obtain molecular identification of these very species from the Brazilian coast. According to Hebert *et al.* (2003), from the “Consortium for the Barcode of Life” (Ratnasingham and Hebert, 2007), divergence in COI sequences consistently facilitates the discrimination of closely allied species in all animal phyla, except the Cnidaria. The use of molecular identification of oyster stocks should facilitate monitoring the distribution of native and exotic species, both in the wild and in culture.

## Materials and Methods

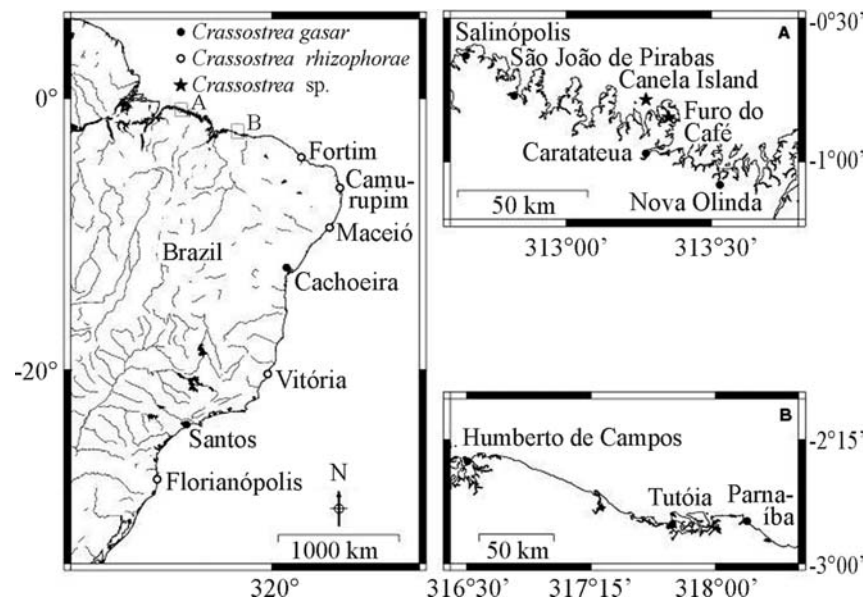
Samples of *Crassostrea gasar* and *C. rhizophorae*, previously identified by means of rRNA 16S DNA sequences (Varela *et al.*, 2007), were compared to those published by Lapègue *et al.* (2002) in the GenBank (AJ312937 and AJ312938, respectively), and to the sequence of *C. brasiliiana* (= *C. gasar*) deposited in the GenBank (DQ839413) by Pie *et al.* (2006). In order to verify the existence of *Crassostrea paraibanensis*, the rRNA 16S gene of five individuals from the Paraíba river estuary (Paraíba state, Brazil) were sequenced. The results showed that these sequences were identical to those of *C. rhizophorae* published by Varela *et al.* (2007). Therefore, COI genes of oysters from the Paraíba river estuary were not sequenced.

The samples of *C. gasar* (n = 215) and *C. rhizophorae* (n = 67) included in our analyses were collected from nine and five localities, respectively (Table 1, Figure 1). Oysters were also collected at Vila Lauro Sodré (00°51'11.2" S, 47°53'24.7" W; municipality of Curuçá, Pará state). Preliminary studies (rRNA16S) revealed these to be *C. gasar* (unpublished data). Young specimens (n = 10; < 2 months old) of a third unidentified *Crassostrea* sp. (n = 10), were obtained from plastic spat collectors at two sites on Canela Island, in the municipality of Bragança (00°47'02" S, 46°43'32.9" W), as well as from a wooden bridge on the Furo do Café tidal channel (00°50'43" S, 46°38'50" W). The scientific names and GenBank sequence accession numbers of oysters compared in the present study are described in Tables 1 and 2. *Crassostrea* sp. collected from Bragança is referred to as *Crassostrea* sp. Canela, the first site where this oyster was found by the authors.

DNA was extracted from the adductor muscle, according to the protocol of Sambrook *et al.* (1989). *Crassostrea rhizophorae* COI sequences were obtained by direct sequencing of PCR amplified fragments, using the primers described by Folmer *et al.* (1994). Samples of *Crassostrea gasar* and *Crassostrea* sp. Canela could only be amplified with a pair of primers designed by C. H. Tagliaro (LCOC-CG-1490 5'- TGTCAACAAATCATT TAGACATTGG-3' and HCOC-CG-2190 5'- TACTTGA CCAAAAACATAAGACATGA-3'), based on the mitochondrial genome sequence of *Crassostrea gigas* (GenBank: NC\_001276). The reaction protocol for the samples consisted of initial denaturing at 95 °C for 3 min; 35 cycles of 1 min at 95 °C, 1 min at 45 °C (*C. gasar*),

**Table 1** - Species, sample size (n), number of COI haplotypes (H), sampling location and GenBank accession number of Brazilian oysters used in the present study.

| Species                       | n                              | H | Municipality (Locality)      | GenBank accession number   |
|-------------------------------|--------------------------------|---|------------------------------|--|
| <i>Crassostrea gasar</i>      | 25                             | 7 | Salinópolis                  | HM003499, HM003501, HM003504, HM003507, HM003509, HM003512, HM003513           |
|                               | 32                             | 7 | São João de Pirabas          | HM003499, HM003502, HM003503, HM003504, HM003505, HM003506, HM003507           |
|                               | 30                             | 5 | Bragança (Caratateua)        | HM003499, HM003504, HM003508, HM003509, HM003510                               |
|                               | 30                             | 3 | Augusto Corrêa (Nova Olinda) | HM003499, HM003500, HM003501   |
|                               | 11                             | 4 | Humberto Campos              | HM003499, HM003517, HM003518, HM003519   |
|                               | 15                             | 4 | Tutóia                       | HM003499, HM003507, HM003515, HM003516   |
|                               | 20                             | 7 | Parnaíba                     | HM003499, HM003516, HM003520, HM003521, HM003522, HM003523, HM003524           |
|                               | 30                             | 4 | Cachoeira                    | HM003499, HM003504, HM003507, HM003511   |
|                               | 22                             | 2 | Santos                       | HM003504, HM003514   |
|                               | <i>Crassostrea rhizophorae</i> | 9 | 6                            | Fortim   |
| 17                            |                                | 8 | Camurupim                    | HM003475, HM003476, HM003493, HM003494, HM003495, HM003496, HM003497, HM003498 |
| 16                            |                                | 8 | Maceió                       | HM003475, HM003476, HM003484, HM003485, HM003486, HM003487, HM003488, HM003489 |
| 14                            |                                | 6 | Vitória                      | HM003475, HM003476, HM003480, HM003481, HM003482, HM003483                     |
| 11                            |                                | 5 | Florianópolis                | HM003475, HM003476, HM003477, HM003478, HM003479                               |
| <i>Crassostrea</i> sp. Canela | 7                              | 2 | Bragança (Canela Island)     | HM003525, HM003526   |
|                               | 3                              | 1 | Bragança (Furo do Café)      | HM003525   |

**Figure 1** - Map of Brazil indicating the sampling locations for mangrove oysters.

45.5 °C (*C. rhizophorae*) or 41.4 °C (*Crassostrea* sp. Canela), and 90 s at 72 °C, followed by a final extension at 72 °C for 7 min. The PCR products were purified using ExoSAP-IT® (Pharmacia). DNA sequences were obtained on both strands using dye terminator cycle sequencing re-

actions (ABI Prism Dye Terminator Cycle Sequencing Ready Reaction, Applied Biosystems), that were subsequently loaded onto an automatic sequencer (Applied Biosystems model 377), according to manufacturer's protocols.

Sequence alignment was carried out with the BioEdit 7 (Hall, 1999) and Clustal X 1.82 (Thompson *et al.*, 1997) programs. Only distinct COI sequences of *C. gasar*, *C. rhizophorae* and *Crassostrea* sp. Canela were aligned with sequences of the other species obtained from the GenBank. Nucleotide frequencies and transition/transversion ratios were obtained by means of Mega 4.0.2 software (Tamura *et al.*, 2007). A saturation test was carried out with the DAMBE 4.2.13 program (Xia and Xie, 2001). A set of aligned sequences is considered to be phylogenetically informative if the observed substitution saturation index (*Iss*) is significantly lower than the critical value of *Iss* (Xia and Xie, 2001). Phylogenetic analyses were undertaken with PAUP\* 4.0b10 (Swofford, 2002), using neighbor-joining (NJ) and maximum parsimony (MP), and with PHYML version 3 (Guindon and Gascuel, 2003) using maximum likelihood (ML) methods. MODELTEST 3.07 (Posada and Crandall, 1998) was used for choosing the best model for use in NJ and ML analyses through Hierarchical Likelihood Ratio Tests (HLRTs). Heuristic search was applied in MP and NJ analyses. The robustness of phylogenetic hypotheses obtained, were tested by bootstrapping (Felsenstein, 1985) with 1000 pseudo-replicates for ML and 2000 for NJ and MP. The criterion adopted to evaluate robustness was to consider bootstrap values equal or superior to 90% as being informative.

Two sets of sequence alignments were carried out. For the first set (Analysis I), use was made of only one partial COI sequence of *C. rhizophorae*, one of *C. gasar*, two of *Crassostrea* sp. Canela, and sequences collected from the GenBank: 10 different species of *Crassostrea*, five of *Ostrea*, one of *Ostreola* and one of *Saccostrea*, besides two Lophinae as outgroups (Table 2). For the second set (Analysis II), use was made of 52 different sequences of COI from three Brazilian species of oysters (Table 1), and sequences collected from the GenBank: 10 species of *Crassostrea*, one sequence of *Saccostrea cucullata* (AY038076), with sequences of *Ostrea chilensis* (AF112286) and *Ostrea edulis* (AF120651) as outgroups (Table 2). Analysis I was undertaken to verify whether *Crassostrea* was a monophyletic group, and Analysis II to avoid any influence from saturation that might be caused by Lophinae sequences in the analysis of *Crassostrea* species, as well as to reduce the number of sequences in the analysis itself.

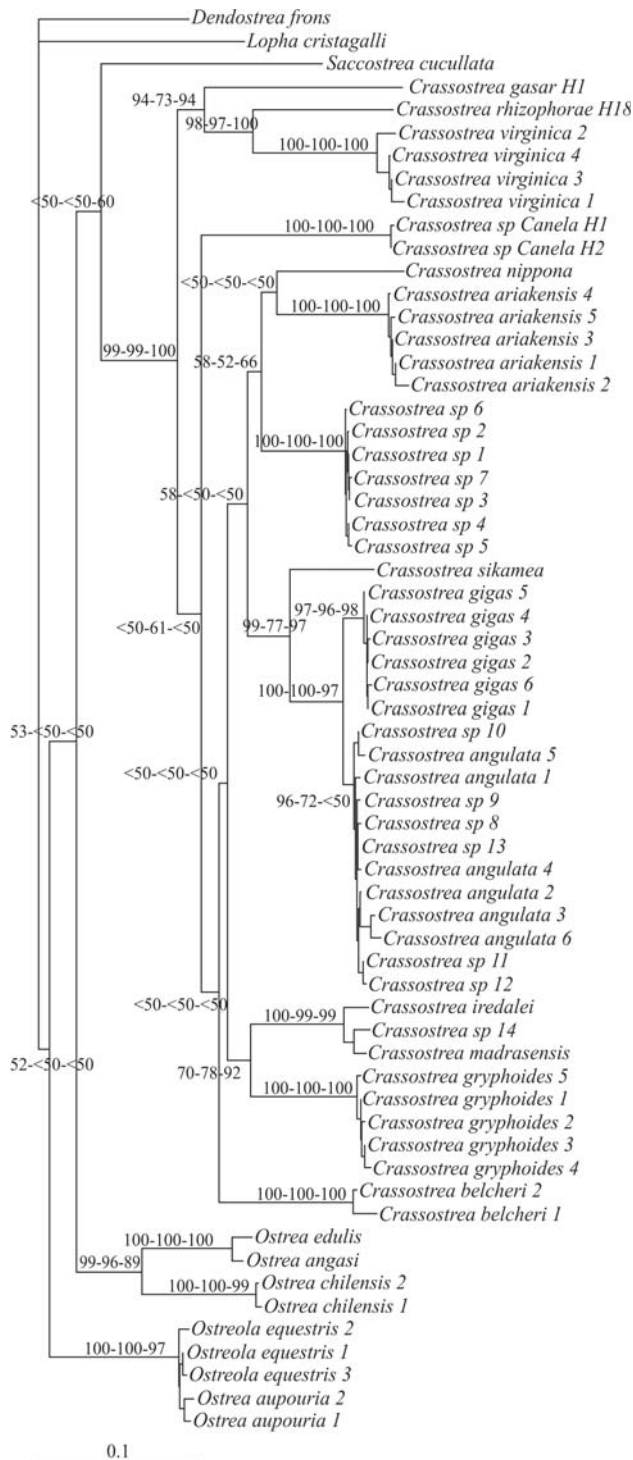
## Results

The divergence in COI sequences was compatible with the presence of three different species of *Crassostrea* along the Brazilian coast. *C. gasar* (= *C. brasiliiana*) was widespread and, in the present study, was found and genetically identified from Vila Lauro Sodré (00°51'11.2" S, 47°53'24.7" W; in the municipality of Curuçá, Pará state) to Santos (São Paulo state). *C. rhizophorae* was found from Fortim to Florianópolis, although some small specimens

**Table 2** - GenBank accession numbers of COI sequences of species of Ostreinae and Lophinae used in the analyses.

| Subfamily                        | Species                             | GenBank accession numbers |
|----------------------------------|-------------------------------------|---------------------------|
| Ostreinae                        | <i>Crassostrea rhizophorae</i> H18* | HM003492 <sup>1</sup>     |
|                                  | <i>Crassostrea gasar</i> H1*        | HM003499 <sup>1</sup>     |
|                                  | <i>Crassostrea</i> sp1 - Canela*    | HM003525                  |
|                                  | <i>Crassostrea</i> sp2 - Canela*    | HM003526                  |
|                                  | <i>Crassostrea angulata</i> 1*      | AF152567 <sup>2</sup>     |
|                                  | <i>Crassostrea angulata</i> 2*      | AJ553907 <sup>2</sup>     |
|                                  | <i>Crassostrea angulata</i> 3*      | AJ553908 <sup>2</sup>     |
|                                  | <i>Crassostrea angulata</i> 4*      | AY397685 <sup>2</sup>     |
|                                  | <i>Crassostrea angulata</i> 5*      | AY397686 <sup>2</sup>     |
|                                  | <i>Crassostrea angulata</i> 6*      | AY455664 <sup>2</sup>     |
|                                  | <i>Crassostrea ariakensis</i> 1*    | AF152569 <sup>3</sup>     |
|                                  | <i>Crassostrea ariakensis</i> 2*    | AF300617 <sup>3</sup>     |
|                                  | <i>Crassostrea ariakensis</i> 3*    | AY160752 <sup>3</sup>     |
|                                  | <i>Crassostrea ariakensis</i> 4*    | AY160753 <sup>3</sup>     |
|                                  | <i>Crassostrea ariakensis</i> 5*    | AY160754 <sup>3</sup>     |
|                                  | <i>Crassostrea belcheri</i> 1*      | AY038077 <sup>3</sup>     |
|                                  | <i>Crassostrea belcheri</i> 2*      | AY160755 <sup>3</sup>     |
|                                  | <i>Crassostrea gigas</i> 1          | AB033687 <sup>3</sup>     |
|                                  | <i>Crassostrea gigas</i> 2*         | AF152565 <sup>3</sup>     |
|                                  | <i>Crassostrea gigas</i> 3*         | AF280608 <sup>3</sup>     |
|                                  | <i>Crassostrea gigas</i> 4*         | AJ553909 <sup>3</sup>     |
|                                  | <i>Crassostrea gigas</i> 5*         | AJ553910 <sup>3</sup>     |
|                                  | <i>Crassostrea gigas</i> 6*         | AJ553911 <sup>3</sup>     |
|                                  | <i>Crassostrea iredalei</i> *       | AY038078 <sup>3</sup>     |
|                                  | <i>Crassostrea nippona</i> *        | AF300616 <sup>3</sup>     |
|                                  | <i>Crassostrea sikamea</i> *        | AF152568 <sup>3</sup>     |
|                                  | <i>Crassostrea</i> sp 1*            | AJ553912 <sup>4</sup>     |
|                                  | <i>Crassostrea</i> sp 2*            | AY160746 <sup>4</sup>     |
|                                  | <i>Crassostrea</i> sp 3*            | AY160747 <sup>4</sup>     |
|                                  | <i>Crassostrea</i> sp 4*            | AY160748 <sup>4</sup>     |
|                                  | <i>Crassostrea</i> sp 5*            | AY160749 <sup>4</sup>     |
|                                  | <i>Crassostrea</i> sp 6*            | AY160750 <sup>4</sup>     |
|                                  | <i>Crassostrea</i> sp 7*            | AY160751 <sup>4</sup>     |
|                                  | <i>Crassostrea</i> sp 8*            | AY249023 <sup>4</sup>     |
|                                  | <i>Crassostrea</i> sp 9*            | AY249024 <sup>4</sup>     |
|                                  | <i>Crassostrea</i> sp 10*           | AY249025 <sup>4</sup>     |
|                                  | <i>Crassostrea</i> sp 11*           | AY249027 <sup>4</sup>     |
|                                  | <i>Crassostrea</i> sp 12*           | AY249031 <sup>4</sup>     |
|                                  | <i>Crassostrea</i> sp 13*           | AY249032 <sup>4</sup>     |
|                                  | <i>Crassostrea</i> sp 14*           | AY249033 <sup>4</sup>     |
|                                  | <i>Crassostrea virginica</i> 1*     | AF152566 <sup>1</sup>     |
|                                  | <i>Crassostrea virginica</i> 2*     | AY376633 <sup>1</sup>     |
|                                  | <i>Crassostrea virginica</i> 3*     | AY376634 <sup>1</sup>     |
|                                  | <i>Crassostrea virginica</i> 4*     | AY376635 <sup>1</sup>     |
|                                  | <i>Crassostrea gryphoides</i> 1*    | EU007489 <sup>3</sup>     |
|                                  | <i>Crassostrea gryphoides</i> 2*    | EU007491 <sup>3</sup>     |
|                                  | <i>Crassostrea gryphoides</i> 3*    | EU007488 <sup>3</sup>     |
| <i>Crassostrea gryphoides</i> 4* | EU007487 <sup>3</sup>               |                           |
| <i>Crassostrea gryphoides</i> 5* | EU007486 <sup>3</sup>               |                           |
| <i>Crassostrea madrasensis</i> * | EU007462 <sup>3</sup>               |                           |
| <i>Ostrea angasi</i>             | AF540598                            |                           |
| <i>Ostrea aoupouria</i> 1        | AY376630                            |                           |
| <i>Ostrea aoupouria</i> 2        | AY376632                            |                           |
| <i>Ostrea chilensis</i> 1*       | AF112286                            |                           |
| <i>Ostrea chilensis</i> 2        | AF112289                            |                           |
| <i>Ostrea edulis</i> *           | AF120651                            |                           |
| <i>Ostreola equestris</i> 1      | AY376611                            |                           |
| <i>Ostreola equestris</i> 2      | AY376618                            |                           |
| <i>Ostreola equestris</i> 3      | AY376626                            |                           |
| <i>Saccostrea cucullata</i> *    | AY038076                            |                           |
| Lophinae                         | <i>Lopha cristagalli</i>            | AB076908                  |
|                                  | <i>Dendostrea frons</i>             | AB084109                  |

<sup>1</sup>Atlantic species; <sup>2</sup>Indo-Pacific species introduced into the Atlantic; <sup>3</sup>Indo-Pacific species; <sup>4</sup>Unidentified species. \*Used in Analyses I and II.



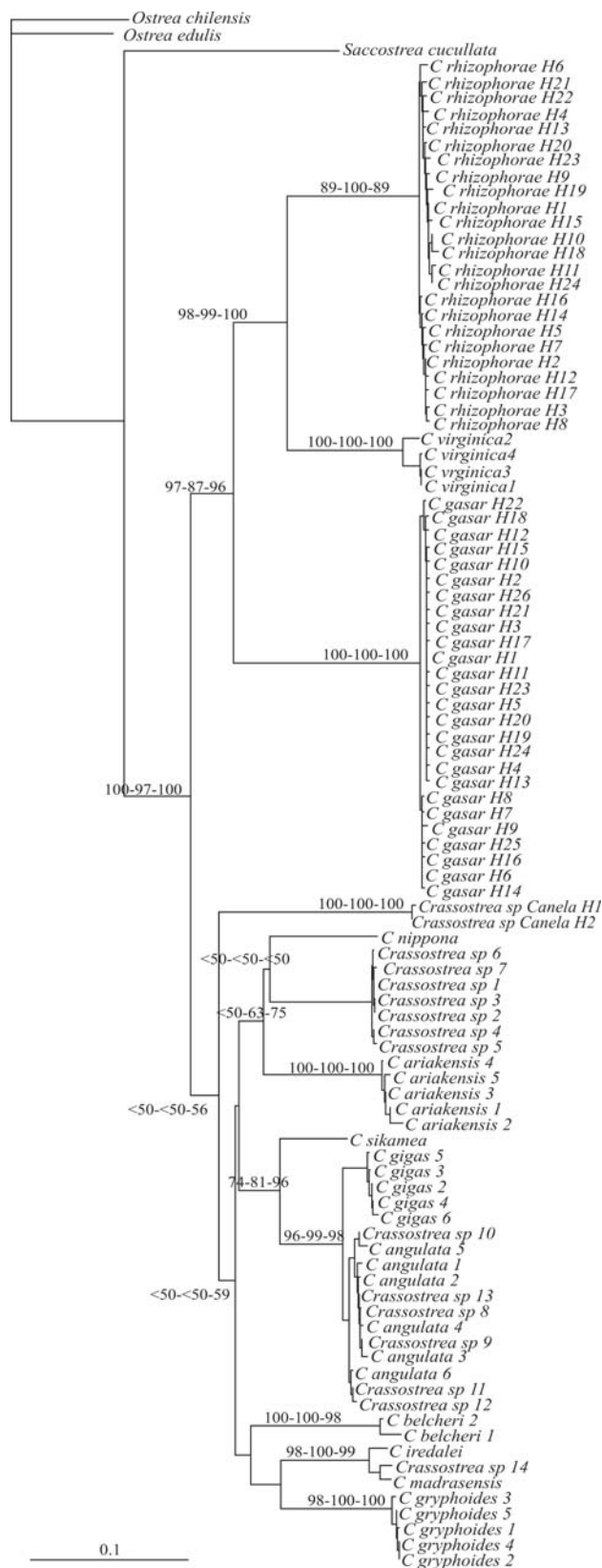
**Figure 2** - Neighbor-joining (NJ) tree for Ostreinae based on COI. *Dendostrea frons* and *Lopho cristagalli* were used as outgroups. Numbers above or below the branches are the bootstrap support values for NJ, Maximum Parsimony and Maximum Likelihood analyses, respectively.

(< 3 cm) were also encountered and genetically identified from Ajuruteua beach (00°50' S, 46°36' W), in the municipality of Bragança. A third species, *Crassostrea* sp. Canela, which could not be identified by molecular GenBank data

comparison, was found only in the municipality of Bragança (Table 1).

The final alignment of Analysis I sequences was composed of 567 sites (corresponding to nucleotides 169 to 735 of NC007175). Only slight saturation ( $I_{ss} = 0.236$ ,  $I_{ss_c} = 0.790$ ;  $p < 0.0001$ ) was detected by saturation testing using COI sequences. The maximum likelihood best fit model for the 62 samples was the General Time-Reversible model – GTR (Lanave *et al.*, 1984; Rodriguez *et al.*, 1990). The settings for the best fit model selected were: base sequences (A = 0.2555, C = 0.1523, G = 0.1860, T = 0.4062); gamma distribution shape parameter ( $\alpha = 0.6664$ ); substitution model rate matrix (Rmat; A-C = 1.2887, A-G = 13.3881, A-T = 1.1207, C-G = 2.1181, C-T = 18.3779, G-T = 1.0000); and proportion of invariable sites (Pinvar = 0.4527). Forty-eight most parsimonious trees were obtained (best tree score = 1176; CI = 0.369; RI = 0.790). Phylogenetic trees based on ML, NJ (Figure 2) and MP, using COI sequences, strongly supported monophyly of the *Crassostrea* genus (bootstrap values: NJ = 99%, MP = 99%, ML = 100%). The *Crassostrea* sp. Canela specimens were grouped inside the *Crassostrea* clade, although without joining the Atlantic group.

In Analysis II, 538 bp (corresponding to nucleotides 181 to 718 of NC007175) were considered. Intraspecific comparison showed *C. gasar* had 26 different COI sequences (H1 to H26), *C. rhizophorae* 24 (H1 to H24) and *Crassostrea* sp. Canela two (H1 and H2). The aligned *Crassostrea* sequences revealed 226 variable sites, of which 211 were parsimony informative. There were no indels. The average nucleotide base frequencies for *Crassostrea* sequences ( $n = 97$ ) were 0.376 (T), 0.189 (C), 0.230 (A) and 0.205 (G), whereas the average transition/transversion rate was 1.49. Little saturation of nucleotide sequences ( $I_{ss} = 0.226$ ,  $I_{ss_c} = 0.798$ ;  $p < 0.0001$ ) was detected by saturation testing using COI sequences. The maximum likelihood best fit model for the 100 samples was the General Time-Reversible model – GTR (Lanave *et al.*, 1984; Rodriguez *et al.*, 1990). The settings for the best fit model selected were: base sequences (A = 0.2585, C = 0.1453, G = 0.1915, T = 0.4047); gamma distribution shape parameter ( $\alpha = 0.7680$ ); substitution model rate matrix (Rmat; A-C = 1.2253, A-G = 14.1526, A-T = 0.9369, C-G = 2.5470, C-T = 16.6255, G-T = 1.0000); and proportion of invariable sites (Pinvar = 0.4752). Divergence matrix values in Atlantic *Crassostrea* oyster species ranged from 0.167 (*C. virginica* x *C. rhizophorae*) to 0.261 (*C. gasar* x *C. rhizophorae*), and in Indo-Pacific oysters from 0.021 (*C. angulata* x *C. gigas*) to 0.219 (*C. gryphoides* x *C. angulata*). Molecular data revealed *Crassostrea* sp. Canela to be more similar to Indo-Pacific oysters ( $d = 0.206$  to 0.244) than to Atlantic ( $d = 0.276$  to 0.309). The most similar *Crassostrea* species to *Crassostrea* sp. Canela was *C. belcheri* ( $d = 0.206$  to 0.223) and the least similar *C. gasar* ( $d = 0.291$  to 0.309). One hundred most-parsimonious-trees



**Figure 3** - Neighbor-joining (NJ) tree for ten *Crassostrea* species and one *Saccostrea cucullata* based on COI. *Ostrea chilensis* and *Ostrea edulis* were used as outgroups. Numbers above the branches are the bootstrap support values for NJ, Maximum Parsimony and Maximum Likelihood analyses, respectively.

were obtained (best tree score = 976; CI = 0.406; RI = 0.908). The three Atlantic *Crassostrea* species (*C. gasar*, *C. virginica* and *C. rhizophorae*) were grouped together with strong support from NJ (Figure 3) and ML (NJ = 97%, MP = 87%, ML = 96%). *C. virginica* and *C. rhizophorae* were clustered (NJ = 98%, MP = 99%, ML = 100%) with *C. gasar* basal. The *Crassostrea* sp. Canela specimens did not group with the Atlantic cupped oysters. In the present study, bootstrap analysis based on COI sequences gave no support to monophyly of the Indo-Pacific species.

### Discussion

As with Varela et al. (2007), we also identified the presence of only three species of *Crassostrea* along the Brazilian coast, *C. gasar* (= *C. brasiliiana*) and *C. rhizophorae*, both with relatively wide distributions, and *Crassostrea* sp. Canela, found only at two locations in the Bragança region. Although *C. gasar* was not found through our sampling at two different locations in Santa Catarina state, Melo et al. (2010) found and genetically identified (16S and ITS-2) *C. brasiliiana* (= *C. gasar*) at Florianópolis Island and, more southerly, in the municipality of Laguna (28°30' S; 48°40' W). *C. gasar* was also found and genetically identified (16S) from Paranaguá Bay (Paraná state) by Lapègue et al. (2002). *Crassostrea paraibanensis*, described by Singarajah (1980), was not encountered in the Paraíba river estuary, as the samples sequenced from this locality were all molecularly identified as *C. rhizophorae*.

As the morphological identification of *Crassostrea* species is difficult and strongly influenced by the environment (Lam and Morton, 2003), and as molecular analyses suggest the presence of an Indo-Pacific oyster in Pará state and also that *C. gasar* and *C. brasiliiana* are synonymous, the urgent need arises for morphological studies to either identify or describe this exotic species, and compare the latter two.

*Crassostrea rhizophorae* has been described from the Caribbean to Uruguay (Rios, 1994). In a recent survey (unpublished data), no *Crassostrea* specimens were encountered from Oiapoque to Macapá (Amapá state). In Brazil, Varela et al. (2007) only came across this oyster from Fortim to Florianópolis, without having access to samples further south. In Pará state, some small specimens (unpublished data) were found at Ajuruteua beach, Bragança. Larvae may have arrived by way of ocean currents and afterwards settled, although post-settlement survival may be brief, possibly through *C. rhizophorae* being poorly adapted to highly variable salinity and warm waters. According to Nascimento (1991), this oyster is adapted to living in salinities between 0 and 40 ppt (the optimum between 7.2 and 28.8 ppt). Furthermore, salinities below 18 ppt are apparently deleterious to gonadal and larval development. The Amazonian coast receives an enormous discharge of fresh water (Ekau and Knoppers, 1999),

especially during the rainy season, with the consequential reduction in salinity and increase in suspended sediment and turbidity (Müller-Karger *et al.*, 1988). At Ajuruteua beach, Bragança, salinity ranged from 10.9 to 40 ppt between January and December, 2003 (Santos-Filho *et al.*, 2008). On the other hand, Lemos *et al.* (1994) showed that the survival of *C. rhizophorae* veligers is primarily determined by temperature, as shown by 100% mortality at 30 °C during their experiment. In 1997, the temperature of the water in the Caeté Bay, Bragança, which usually ranges from 27 °C to 29 °C, presented a minimum of 23 °C in April (wet season) and a maximum of 31 °C in December (dry season) (Camargo and Isaac, 2005).

Intraspecific comparison indicated a large number of different COI sequences for both *C. rhizophorae* and *C. gasar*. *Crassostrea* sp. Canela was shown to have at least two different haplotypes, although there may be more, since the number of specimens was low. The topology of our trees showed that *C. rhizophorae*, *C. virginica*, and *C. gasar* were closely related, thus in agreement with the trees generated by Lapègue *et al.* (2002), Boudry *et al.* (2003), Lam and Morton (2003) and Varela *et al.* (2007), based on the 16S rRNA gene. On the other hand, our results do not strongly support the monophyly of Indo-Pacific oysters, as previously reported by Lam and Morton (2003), Varela *et al.* (2007), and Reece *et al.* (2008), the latter based on COI sequences.

Molecular sequences of *Crassostrea* sp. Canela were very different in comparison with those of native Brazilian cupped oysters (*C. gasar* and *C. rhizophorae*), and were significantly so in comparison with those of other species of *Crassostrea* deposited in the GenBank (Table 1). *Crassostrea* sp. Canela is more similar to Indo-Pacific oysters, particularly *C. belcheri*, a native of Southeast Asia (including the Philippines, Vietnam, Malaysia and Indonesia), and those of India (Carriker and Gaffney, 1996). It is not known in what manner *Crassostrea* sp. Canela arrived at the mangrove coast of Bragança. It may even have been established in the Atlantic Ocean before the emergence of the Isthmus of Panama. On the other hand, an accidental anthropogenic introduction may have occurred during the colonial period (16<sup>th</sup> to 19<sup>th</sup> centuries), when ships belonging to the Portuguese Empire may have brought oysters from the Indo-Pacific to Brazil. The introduction may even have been more recent, via international shipping traffic traveling along the Pará coast towards Manaus, an important industrial and tourist center. Accidental introduction of exotic species has already occurred through either the release of ballast seawater or via external fouling and boring communities on ships (Carlton, 1996). Ó Foighil *et al.* (1998) suggested a similar explanation for the introduction of *C. angulata* to the European coast.

Mechanisms for the introduction of non-indigenous marine organisms were reviewed by Carlton (1989, 1992). Transport by shipping is the most important human activ-

ity, culminating in the introduction of exotic organisms, as fouling and boring species, inside the vessels themselves or in ballast water (Carlton, 1992). Moreover, oyster culture has been cited as one of the outstanding agents of exotic species conveyance, through intercontinental transport of species, as larvae or recently settled juveniles (Carlton, 1989; Eldredge, 1994). Many invaders may drastically affect the abundance of species in the recipient community, and in so doing, modify the agents of selection on these species (Vermeij, 1996). Although the introduction of exotic species is on the increase, mainly due to transport via ship ballast water and sediment (Carlton, 1992), there appears to be a lack of adequate legislation governing introductions in most countries. Such a lack of policy may impose ecological risks from the introduction of non-native species for aquaculture (Naylor *et al.*, 2001). Thus, the genetic monitoring of exotic species should be included in the range of measures for use in controlling introductions.

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## References

- Boudry P, Heurtebise S, Collet B, Cornette F and Gerard G (1998) Differentiation between populations of the Portuguese oyster, *Crassostrea angulata* Lamarck and the Pacific oyster, *Crassostrea gigas* Thunberg, revealed by mtDNA RFLP analysis. *J Exp Mar Biol Ecol* 226:279-291.
- Boudry P, Heurtebise S and Lapègue S (2003) Mitochondrial and nuclear DNA sequence variation of presumed *Crassostrea gigas* and *Crassostrea angulata* specimens: A new oyster species in Hong Kong? *Aquaculture* 228:15-25.
- Camargo M and Isaac V (2005) Reproductive biology and spatio-temporal distribution of *Stellifer rastrifer*, *Stellifer naso* and *Macrodon ancylodon* (Scianidae) in the Caeté estuary, Northern Brazil. *Braz J Oceanogr* 53:13-21.
- Carlton JT (1989) Man's role in changing the face of the ocean: Biological invasions and implications for conservation of near-shore environments. *Conserv Biol* 3:265-273.

- Carlton JT (1992) Introduced marine and estuarine mollusks of North America: An end-of-the-20th-century perspective. *J Shellfish Res* 11:489-505.
- Carlton JT (1996) Pattern, process, and prediction in marine invasion ecology. *Biol Conserv* 78:97-106.
- Carriker MR and Gaffney PM (1996) A catalogue of selected species of living oysters (Ostreacea) of the world. In: Kennedy VS, Newell RIE and Eble AF (eds) *The Eastern Oyster: Crassostrea virginica*. Maryland Sea Grant, Maryland, pp 1-18.
- Day AJ, Visootviseth P and Hawkins AJS (2000) Genetic diversity among cultured oysters (*Crassostrea spp.*) throughout Thailand. *Sci Asia* 26:115-122.
- Ekau W and Knoppers B (1999) An introduction to the pelagic system of the North-East and East Brazilian shelf. *Arch Fish Mar Res* 47:113-132.
- Felsenstein J (1985) Confidence limits on phylogenies: An approach using the bootstrap. *Evolution* 39:783-791.
- Folmer OM, Black W, Hoeh R, Lutz R and Vrijenhoek R (1994) DNA primers for amplification of mitochondrial cytochrome c oxidase subunit I from diverse metazoan invertebrates. *Mol Mar Biol Biotech* 3:294-299.
- Guindon S and Gascuel O (2003) A simple, fast, and accurate algorithm to estimate large phylogenies by maximum likelihood. *Syst Biol* 52:696-704.
- Gunter G (1950) The generic status of living oysters and the scientific name of the common American species. *Am Midl Nat* 43 438-449.
- Hall TA (1999) BioEdit: A user-friendly biological sequence alignment editor and analysis program for 95/98/NT. *Nucleic Acids Symp Ser* 41:95-98.
- Hebert PDN, Ratnasingham S and deWaard JR (2003) Barcoding animal life: Cytochrome c oxidase subunit I divergences among closely related species. *Proc R Soc Lond B* 270:S96-S99.
- Huvet A, Lapègue S, Magoulas A and Boudry P (2000) Mitochondrial and nuclear DNA phylogeography of *Crassostrea angulata*, the Portuguese oyster endangered in Europe. *Conserv Genet* 1:251-262.
- Ignacio BL, Absher TM, Lazoski AM and Solé-Cava AM (2000) Genetic evidence of the presence of two species of *Crassostrea* (Bivalvia, Ostreidae) on the coast of Brazil. *Mar Biol* 136:987-991.
- Lam K and Morton B (2003) Mitochondrial DNA and morphological identification of a new species of *Crassostrea* (Bivalvia, Ostreidae) cultured for centuries in the Pearl River Delta, Hong Kong, China. *Aquaculture* 228:1-13.
- Lanave C, Preparata G, Saccone C and Serio G (1984) A new method for calculating evolutionary substitution rates. *J Mol Evol* 20:86-93.
- Lapègue S, Boutet I, Leitão A, Heuvertebise S, Garcia P, Thiriott-Quévieux C and Boudry P (2002) Trans-Atlantic distribution of mangrove oyster species revealed by 16S mtDNA and karyological analyses. *Biol Bull* 202:232-242.
- Lemos MBN, Nascimento IA, Araujo MMS, Pereira SA, Bahia I and Smith DH (1994) The combined effects of salinity, temperature, antibiotic and aeration on larval growth and survival of the mangrove oyster, *Crassostrea rhizophorae*. *J Shellfish Res* 13:187-192.
- Melo CMR, Silva FC, Gomes CHAM, Solé-Cava AM and Lazoski C (2010) *Crassostrea gigas* in natural oyster banks in southern Brazil. *Biol Invasions* 12:441-449.
- Müller-Karger FE, McClain CR and Richardson PL (1988) The dispersal of the Amazon's water. *Nature* 333:56-59.
- Nascimento IA (1991) *Crassostrea rhizophorae* (Guilding) and *C. brasiliensis* (Lamarck) in South and America Central. In: Menzel W (ed) *Estuarine and Marine Bivalve Mollusk Culture*. CRC Press, Boca Raton, pp 125-134.
- Naylor RS, Williams SL and Strong D (2001) Aquaculture – A gateway for exotic species. *Science* 294:1655-1656.
- Ó Foighil D, Gaffney PM, Wilbur AE and Hilbish TJ (1998) Mitochondrial cytochrome oxidase I gene sequences support an Asian origin for the Portuguese oyster *Crassostrea angulata*. *Mar Biol* 131:497-503.
- Posada D and Crandall KA (1998) Modeltest: Testing the model of DNA substitution. *Bioinformatics* 14:817-818.
- Pie MR, Ribeiro RO, Boeger WA, Ostrensky A, Falleiros RM and Angelo L (2006) A simple PCR-RFLP method for the discrimination of native and introduced oyster species (*Crassostrea brasiliensis*, *C. rhizophorae* and *C. gigas*; Bivalvia, Ostreidae) cultured in Southern Brazil. *Aquac Res* 37:1598-1600.
- Reece KS, Cordes JF, Stubbs JB, Hudson KL and Francis EA (2008) Molecular phylogenies help resolve taxonomic confusion with Asian *Crassostrea* oyster species. *Mar Biol* 153:709-721.
- Ratnasingham S and Hebert PDN (2007) BOLD: The Barcode of Life Data System (<http://www.barcodinglife.org>). *Mol Ecol Notes* 7:355-364.
- Rios EC (1994) *Seashells of Brazil*. 2nd edition. Fundação Universidade do Rio Grande, Rio Grande, 492 pp.
- Rodriguez FJ, Oliver JL, Marin A and Medina JR (1990) The general stochastic model of nucleotide substitution. *J Theor Biol* 142:485-501.
- Sambrook J, Fritsch E and Maniatis T (1989) *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor Laboratory Press, New York.
- Santos-Filho C, Tagliaro CH and Beasley CR (2008) Seasonal abundance of the shipworm *Neoteredo reynei* (Bivalvia, Teredinidae) in mangrove driftwood from a northern Brazilian beach. *Iheringia Sér Zool* 98:17-23.
- Singarajah KV (1980) On the taxonomy, ecology and physiology of a giant oyster, *Crassostrea paraibanensis*, a new species. *B Mar Sci* 30:833-847.
- Swofford DL (2002) PAUP\*. Version 4.0b10: Phylogenetic analysis using parsimony (\*and other methods). Sinauer Associates, Sunderland.
- Tamura K, Dudley J, Nei M and Kumar S (2007) MEGA 4: Molecular Evolutionary Genetics Analysis. *Mol Biol and Evol* 24:1596-1599.
- Thompson JD, Gibson TJ, Plewniak F, Jeanmougin F and Higgins DG (1997) The Clustal X windows interface: Flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Res* 24:4876-4882.
- Varela ES, Beasley CR, Schneider H, Sampaio I, Marques-Silva NS and Tagliaro CH (2007) Molecular phylogeny of mangrove oysters (*Crassostrea*) from Brazil. *J Molluscan Stud* 73:229-234.
- Vermeij GJ (1996) An agenda for invasion biology. *Biol Conserv* 78:3-9.



Xia X and Xie Z (2001) DAMBE: Data analysis in molecular biology and evolution. *J Hered* 92:371-373.

### Internet Resources

Eldredge LG (1994) Introductions of Commercially Significant Aquatic Organisms to the Pacific Islands. South Pacific Commission, Noumea, New Caledonia, 127 pp.  
<http://www.spc.int/coastfish/Reports/IFRP/Intro/Eldredge.pdf> (October 7, 2009).

Littlepage JL and Poli CR (1999) Oyster culture in the state of Santa Catarina, Brazil. Final Report of the Shellfish Technology Transfer Program (STTP). Blue Water Aquaculture Ltda, Florianópolis, 27 pp.  
<http://www.bwaaquaculture.com> (April 24, 2007).

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