Aquaculture Research

Aquaculture Research, 2013, **44**, 1589–1601 doi: 10.1111/j.1365-2109.2012.03166.x

Molecular identification and distribution of mangrove oysters (*Crassostrea*) in an estuarine ecosystem in Southeast Brazil: implications for aquaculture and fisheries management

Márcia S N Galvão[1,2], Orlando M Pereira[1] & Alexandre W S Hilsdorf[2]

[1]Centro Avançado de Pesquisa Tecnológica do Agronegócio do Pescado Marinho, Instituto de Pesca, Santos, Brasil

[2]Universidade de Mogi das Cruzes, Núcleo Integrado de Biotecnologia, Laboratório de Genética de Organismos Aquáticos e Aquicultura, Mogi das Cruzes, Brasil

Correspondence: A W S Hilsdorf, Universidade de Mogi das Cruzes, Núcleo Integrado de Biotecnologia, Av. Dr. Cândido Xavier de Almeida Souza, 200. 08780-911, Mogi das Cruzes, SP, Brazil. E-mail: wagner@umc.br

Abstract

This study investigated the existing species of *Crassostrea* in the natural environment, farming systems and artificial spat collectors at a protected estuarine area in Brazil, through PCR-RFLP of mitochondrial 16S rRNA. Among 450 samples collected in the natural environment, 303 were *C. brasiliana* and 147 *C. rhizophorae*. Oysters present in the rocky subtidal zone were *C. brasiliana*. However, both species occurred on mangrove roots in the intertidal zone. Farm-raised samples included only *C. brasiliana*. It was observed that attached specimens in commercial collectors had a banding pattern distinct from *C. brasiliana* and *C. rhizophorae*, indicating the presence of a third species in the estuary. The 16S rRNA sequence analysis showed that these specimens are clustered with the oysters from Pacific and Indian Oceans, and genetically close to the oysters of Beihai, China (0.3% genetic distance). Oysters obtained from the seed capture showed 17.8% distance of in relation to *C. brasiliana*, 17.6% for *C. rhizophorae* and 10.3% for *C. gigas*, demonstrating high genetic divergence from these species. The occurrence of an exotic species in the Cananéia estuary may have strong ecological and economic implications which require new guidelines for farming, conservation and sustainable fisheries management for the native oyster species.

Keywords: Cananéia estuary, Brazil, *Cassostrea*, mtDNA, 16S rRNA, Ostreidae, species identification

Introduction

Oysters are important fishery resources for coastal communities. In Brazil, representatives of the Ostreidae family include the genera *Ostrea* Linnaeus (1758), *Crassostrea* Sacco (1897) and *Lopha* Rölding (1798) (Rios 1994). Oysters of economic interest include the *Crassostrea* genus, which are distributed along the Brazilian coast, mainly in estuarine regions. Typically, they attach to rhizophores of mangrove trees from the species *Rhizophora mangle* L. in the intertidal zone and on consolidated substrates in the subtidal zone (Rios 1994).

Identification based on shell characteristics, such as colour, shape, structure and insertion of the adductor muscle is extremely prone to error (Ignacio, Absher, Lazoski & Solé-Cava 2000) as oyster morphology can be strongly influenced by environmental conditions. Therefore, molecular methods are essential in establishing differences between species (Thorpe & Solé-Cava 1994).

In this regard, several studies have been performed worldwide to clarify the taxonomic differences among oyster species (Lam & Morton 2006; Reece, Cordes, Stubbs, Hudson & Francis 2008; Wang, Guofan, Liu & Guo 2008; Liu, Li, Kong, Yu & Zheng 2011). Polymerase chain reaction–based restriction fragment length polymorphism (PCR-RFLP) analyses have been successfully used in taxonomic studies of oysters (Boudry, Heurtebise, Collet, Cornette & Gérard 1998; Klinbunga, Khamnamtong, Puanglarp, Jarayabhand,

Yoosukh & Menasveta 2005; Xia, Yu & Kong 2009). Thus, studies involving mitochondrial and nuclear DNA have been conducted to identify Brazilian oysters (Lapègue, Boutet, Leitão, Heurtebise, Garcia, Thiriot-Quiévreux & Boudry 2002; Pie, Ribeiro, Boeger, Ostrensky, Falleiros & Angelo 2006; Varela, Beasley, Schneider, Sampaio, Marques-Silva & Tagliaro 2007; Melo, Silva, Gomes, Solé-Cava & Lazoski 2010a; Melo, Varela, Beasley, Schneider, Sampaio, Gaffney, Reece & Tagliaro 2010b; Lazoski, Gusmão, Boudry & Solé-Cava 2011).

In Cananéia estuary, several studies have investigated the cultivation and integrity of oyster banks (Akaboshi & Pereira 1981; Pereira & Chagas Soares 1996; Pereira, Henriques & Machado 2003), using *C. brasiliana* as a synonym of *C. rhizophorae* according to Rios (1994).

These molecular studies revealed the existence of two or more oyster species of the genus *Crassostrea* on the Brazilian coast: *C. brasiliana* (Lamarck) and *C. rhizophorae* (Guilding) being distinct. They concluded that *C. brasiliana* is not a junior synonym of *C. rhizophorae* as previously described by Rios (1994). Otherwise, Lapègue *et al.* (2002) recorded *C. gasar* (Andanson) (species from the Atlantic coast of Africa) along the Brazilian coast. According to a recent study carried out by Lazoski *et al.* (2011), it seems that *C. brasiliana* and *C. gasar* might be synonym names for the same species.

The lagoon estuarine system of Cananéia, Iguape and Paranaguá is located on the southeastern coast of Brazil. Due to its ecological importance, this ecosystem is considered as the third most productive in the South Atlantic. It also has the largest natural mangrove oyster bank in the southeast and south of the country. Therefore, the region has been declared a World Natural Heritage site for scientific knowledge and conservation of human values based on sustainable development standards being part of the Atlantic Forest Biosphere Reserve (UNESCO 1999, 2005).

Given the evidence of two or more species of the genus *Crassostrea* occurring along the Brazilian coast, it has become imperative to correctly identify the oyster species of the Cananéia estuary and the subenvironments in which they occur, once this will allow adequate management of extraction and farming activities for conservation of this resource.

This study had the following objectives: (1) discriminating among the species that occur in the Cananéia estuary and determining their spatial distribution; (2) identifying the predominant species farmed in the region and in artificial seed collectors.

Materials and methods

Sampling

Oysters larger than 5 cm were collected from five locations in the Cananéia estuary and one in the Guaraú River, Peruíbe, State of São Paulo, Brazil (Fig. 1, Table 1). At each collection site in the Cananéia estuary, oysters from intertidal zones attached on mangrove roots, among which were included those called 'parangas' by the local fishermen due to their reduced growth, and oysters in the subtidal zone attached on rocky substrates were obtained. In Guaraú River, oysters were collected by diving at 4–7 m depth. Oysters in the rocky subtidal zone were called ST, those attached on mangrove roots in the intertidal zone were called IT and those popularly known as 'parangas' were called PIT.

For juveniles sampling, spat collectors were released at the site 5 (Table 1) after detection of spat settlement peaks according to the methodology described by Akaboshi and Pereira (1981). Vertical commercial collectors were placed in the subtidal zone of the estuary in two seasons from December 2008 to April 2009 and from December

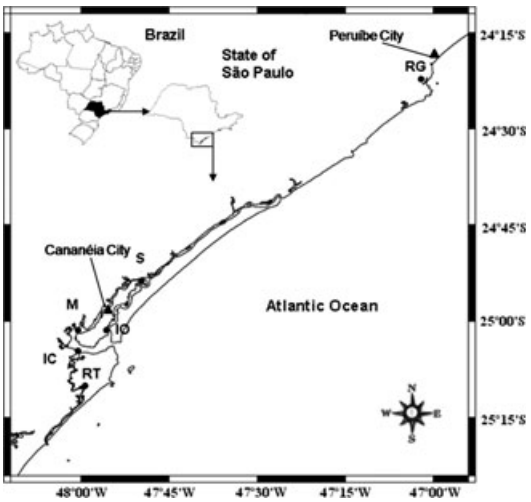

Figure 1 Map of the Cananéia estuarine-lagoon region (25ºS, 48°W), São Paulo, indicating the sampling sites (S, Suiço; M, Mandira; RT, Rio Tapera; IC, Ilha da Casca; IO, Instituto Oceanográfico; RG, Rio Guaraú).

Table 1 Description of the sampling sites for oysters of the genus *Crassostrea* in the estuarine-lagoon region of Cananéia and in the Guaraú River, Peruíbe, and the number of collected specimens

	Sites	Geographic coordinates	Salinity g L^{-1}	Number of specimens	Oysters batches*
1.	Suíço (S)	24°53′31.36″S47°50′14.18″W	0–10	100	Intertidal (IT, PIT) and subtidal oysters (ST)
2.	Mandira (M)	25°01′20.53″S48°00′53.83″W	12–20	133	Intertidal (IT, PIT) and subtidal oysters (ST)
3.	Rio Tapera (RT)	25°10′30.28″S47°59′50.26″W	13–17	150	Intertidal (IT, PIT) and subtidal oysters (ST)
4.	Ilha da Casca (IC)	25°05′19.53″S48°00′53.40″W	28–32	150	Intertidal (IT, PIT) and subtidal oysters (ST)
5.	Instituto Oceanográfico (IO)	25°01′12.03″S47°55′28.35″W	25–32	150	Intertidal (IT, PIT) and subtidal oysters (ST)
6.	Rio Guaraú (RG)	24°23′01.00″S47°02′29.79″W	–	50	Subtidal oysters (ST)
	Total			733	

*IT, intertidal oysters batches; PIT, intertidal oysters batches known as 'parangas'; ST, subtidal oysters batches.

2009 to February 2010 at 5 m depth. After 2 months for spat growth, collectors were removed from the water and the juvenile oysters were collected.

The farmed oysters were obtained from the Cananéia Oyster Producers Cooperative. These oysters had been raised on trays placed on the intertidal zone during 4 months.

Biometrics

Oysters were maintained in isothermal boxes and transported to the laboratory to obtain biometric data by measuring height (dorsal-ventral axis), length (anterior-posterior axis) and width (lateral axis) of each oyster (Galtsoff 1964) with the aid of a calliper with 0.01 mm accuracy. A total of 983 oysters were measured being 783 adults (50 from cultivation) and 200 juveniles from commercial collectors.

PCR-RFLP of partial 16S ribosomal subunit of mitochondrial DNA

The minimum sample size to use in PCR-RFLP analysis was determined using the method of Schwager, Mutchler, Federer and Scully (1993), according to the expression: $n = \ln(1-\beta)/\ln(1-P)$, where β is the probability to observe a desired haplotype (0.95) and P is the probability to not observe a desired haplotype (0.05).

A total of 700 samples were analysed using PCR-RFLP. Ninety oysters from each location (60 attached on mangrove roots and 30 attached on rocky substrates), except for the Suíço location (60 specimens), 30 oysters from Guaraú River, 50 oysters from farming and 200 juveniles from spat collectors (100 from each capture period) were used.

The oysters were opened with the aid of a stainless steel knife. Samples of approximately 1 cm^3 of the adductor muscle were cut with a scalpel and immediately placed in 95% ethanol, then stored in a freezer at −20°C. With juveniles, all internal tissue was removed as it was not possible to remove only the adductor muscle due to its small size and limited amount of muscle tissue.

The DNA extraction was performed using the Illustra tissue and cells genomicPrep Mini Spin Kit (GE Healthcare, Life Sciences, Little Chalfont, Buckinghamshire, England). To species identification, PCR-RFLP on a partial sequence of the mitochondrial gene encoding the largest subunit of rRNA (16S) was used as described by Pie *et al.* (2006).

The amplifications by PCR were performed for a final volume of 20 μL containing 5–20 ng μL^{-1} of DNA; 1.25 U of *Taq* DNA polymerase (Fermentas); 1× buffer solution (KCl); 0.25 mM of dNTPs; 0.5 μM of each primer and 2.5 mM of $MgCl_2$. The oligonucleotide primers used to amplify 16S were 16S.AR (5′-CGCCTGTTTATCAAAAACAT-3′) and 16S.BR (5′-CCGGTCTGAACTCAGATCACGT-3′) (Palumbi, Martin, Romano, Mcmilan, Stice & Grabowski 1991). The DNA amplification reaction was conducted in PTC-200 thermal cycler (MJ Research) with initial cycle of denaturation at 94°C for 4 min, followed by 32 cycles (94°C for 30 s, 56°C for 40 s, 72°C for 1 min) and final extension at 72°C for 1 min.

The amplification products were digested with the *Hae*III endonuclease to search for polymorphism in restriction sites, generating RFLPs. Digestion was conducted at 10 μL final volume, including 0.5 μL of *Hae*III (10 U mL^{-1}), 1 μL of 1× buffer solution (50 mM Tris-HCl pH 8.0, 10 mM $MgCl_2$, 50 mM NaCl) (Invitrogen) and 4 μL of the PCR product. Solutions were incubated for 1–2 h at 37°C in thermal block.

The resulting sizes of restriction fragments were determined in 2.5% agarose gel electrophoresis by comparison with a standard DNA molecular weight marker. Images were digitalized for further analysis of the banding patterns.

Sequence analysis

To confirm the results of the RFLP analysis, 14 samples were sequenced (4 intertidal and 5 subtidal oysters and 5 juveniles from spat collection). Sequences were deposited in GenBank (accession numbers JN849099–JN849112). Additional sequences obtained from GenBank were also included in the analyses (Table 2).

The amplification products of the 16S rRNA fragment were purified with the GFX™ PCR DNA and Gel Band Purification Kit (GE Healthcare Life Sciences), and eluted in ultrapure water. The purified PCR products were sequenced with the BigDye Terminator Cycle Sequencing kit (Applied Biosystems, Carlsbad, CA, USA) according to the protocols provided by the manufacturer. Electrophoresis of the purified samples was performed in ABI Prism 3100 DNA Sequencer (Applied Biosystems). Generated sequences were analysed using *NEBCutter* software to check the restriction sites (Vincze, Posfai & Roberts 2003).

Data analysis

Biometric data were subjected to analysis of variance (ANOVA) to verify the statistical significance among different oyster batches (IT, PIT and ST). Subsequently, the Tukey test was used for means comparisons (Zar 1996). Results of the RFLP analysis were evaluated for the frequency of band patterns generated from each batch sample.

We applied the Chi-square goodness of fit test with Yates's correction for continuity (Zar 1996) to determine the distribution of oyster species in intertidal and subtidal zones, on the bases that the species have the same proportion on different substrates (1:1). This test was also applied to the oysters from intertidal zones attached on mangrove roots called 'parangas' by the local fishermen.

Sequences were aligned with the software Clustal W as implemented in the programme *MEGA 4.1* (Tamura, Dudley, Nei & Kumar 2007). The neighbour-joining method was used to analyse phenetic relationship among samples (Saitou & Nei 1987). Bootstrap re-sampling analysis was performed to assess support for individual nodes using 1000 replicates

Table 2 Accession numbers of sequences deposited in GenBank and previously used for phenetic analysis

Species	Acession number	References
C. angulata	EU815952.1	Xia & Yu 2008
C. ariakensis	AY160757.1	Lam & Morton 2006;
C. brasiliana	DQ839413.1	Pie *et al.* 2006
C. corteziensis	EU733652.1	Perez-Enriquez, Ibarra & Ávila 2008
C. gasar	AJ312937.1	Lapègue *et al.* 2002;
C. gasar	EF473270.1	Varela *et al.* 2007
C. gasar	JF808179.1	Sanchez, Quinteiro, Rey-Mendez, Perez-Martin & Sotelo 2011
C. gigas	DQ839414.1	Pie *et al.* 2006
C. hongkongensis	EU815943.1	Xia & Yu 2008
C. iredalei	EU815954.1	Xia & Yu 2008
C. rhizophorae	DQ839415.1	Pie *et al.* 2006
C. rhizophorae	FJ478030.1	Melo *et al.* 2010a
C. sikamea	EU815951.1	Xia & Yu 2008
C. virginica	AF092285.1	McCarthy, Brown, Johnstone & Wheeler 1998
Crassostrea sp.	EF473280.1	Varela *et al.* 2007
Crassostrea sp.	HQ660984.1	Liu *et al.* 2011
Crassostrea sp.	HQ660985.1	Liu *et al.* 2011
Ostrea edulis	DQ280032.1	Giribet, Okusu, Lindgren, Huff, Schrodl & Nishiguchi 2006

with 10,000 random additions (Nei & Kumar 2000). The genetic diversity was calculated from the Kimura two-parameter (K2P) distances (Kimura 1980).

Species names used in this study followed the terminology described in *Malacolog 4.1.0* (Rosenberg 2009).

Results

Biometric data

The ST oysters were larger than those from the other batches of all locations ($P < 0.01$), whereas the PIT oysters showed the lowest values in height and length (Table 3). Farmed oysters were, on average, 71.7 mm high, 53.4 mm long and 20.1 mm wide. Oysters originating from spat collectors showed a wide variation in size from 8 mm to 36 mm in height.

PCR-RFLP analysis of a partial 16S ribosomal subunit of mitochondrial DNA

The partial 16S rRNA amplicons from 700 samples were subjected to enzymatic digestion with *Hae*III. All individuals showed banding pattern that permitted the differentiation of oysters from the genus *Crassostrea*.

Figure 2 shows the fragments resulting from the enzymatic digestion, which allowed us to differentiate the species. Bands with larger fragments represented individuals of *C. rhizophorae* (lanes 1, 2, 3, 4 and 9), whereas bands with smaller fragments represented individuals of *C. brasiliana* (lanes 5, 6, 7, 10, 11 and 12). The lane 8 in Fig. 2a and the lane 12 in Fig. 2b show an undigested amplicon of the 16S rRNA fragment used as control.

The juveniles obtained from spat collectors showed band sizes close to 400 bp (lanes 4–11), indicating the presence of a third species at the Cananéia estuary (Fig. 2b) that we called as *Crassostrea* sp. In Fig. 2b and c, lanes 1, 2 and 3 are samples of individuals of *C. rhizophorae* used as controls. The farmed oysters showed the same band pattern as *C. brasiliana* (lanes 4, 5, 6, 7, 8 and 9) (Fig. 2c).

Fragments generated by *Hae*III were compared from three oyster species found in the Cananéia estuary with those from *C. gigas* (Thunberg) farmed in the south of Brazil. The species *C. brasiliana*, *C. rhizophorae*, *Crassostrea* sp. and *C. gigas* presented 4, 3, 2 and 2 restriction sites respectively (Table 4). Sizes of largest fragments used for comparison among the three species occurring at the Cananéia estuary were 235, 261 and 391 bp for *C. brasiliana*, *C. rhizophorae* and *Crassostrea* sp. respectively.

Based on RFLP patterns, we observed that of the 450 samples (adult oysters) collected in natural environments, 303 were *C. brasiliana* species and 147 *C. rhizophorae*. All ST oysters individuals attached on rocks were *C. brasiliana*. Both species occurred on mangrove roots of the intertidal zone (IT) at the same proportion ($\chi^2 = 2.315$; $P = 0.1281$; $n = 270$). The proportion of *C. rhizophorae* in the group of intertidal oysters known as 'paranga' by fisherman (PIT) was significantly higher than *C. brasiliana* ($\chi^2 = 66.008$; $P < 0.0001$; $n = 120$).

Based on the *in silico* analysis performed in this study, another enzyme that can be used for oyster diagnostics is *Alu*I (5′… AG | CT… 3′), for which

Table 3 Average values of height, length and width of oysters of the genus *Crassostrea* collected in the subtidal zone (ST) and intertidal zones (IT and PIT) from five sites in the Cananéia estuary (S, M, RT, IC and IO) and one in the Peruíbe estuary (RG)

	Height (mm)						Length (mm)						Width (mm)					
	ST		IT		PIT		ST		IT		PIT		ST		IT		PIT	
Site	AVG	CI	AVG	CI	AVG	CI	AVG	CI	AVG	CI	AVG	CI	AVG	CI	AVG	CI	AVG	CI
1. S	69.1^a	3.9	60.1^b	2.2	–	–	46.2^a	2.8	42.6^b	1.6	–	–	24.3^a	1.7	17.8^b	1.1	–	–
2. M	65.1^a	3.8	58.6^b	2.4	53.8^c	2.8	54.0^a	4.9	42.0^b	1.9	35.8^c	2.5	19.9^a	1.6	18.3^a	1.1	15.8^b	1.5
3. RT	78.3^a	4.0	69.8^b	3.5	60.5^c	2.8	57.4^a	3.1	50.2^b	2.9	39.9^c	1.7	22.1^a	1.3	19.7^b	1.3	17.7^b	1.2
4. IC	78.5^a	3.4	67.4^b	3.0	55.4^c	2.2	60.0^a	2.5	46.3^b	2.5	38.8^c	1.7	25.4^a	1.9	21.8^b	1.6	18.1^c	1.3
5. IO	94.9^a	5.1	58.8^b	2.5	52.4^c	2.2	65.2^a	3.1	41.8^b	2.0	35.7^c	1.8	25.6^a	1.7	9.0^b	1.5	16.8^b	1.2
6. RG	75.4	2.5	–	–	–	–	51.2	2.1	–	–	–	–	26.5	1.3	–	–	–	–

AVG, average values; CI, confidence interval ($P = 0.05$); $n = 50$; average values followed by same letter do not differ by Tukey test ($P < 0.01$).

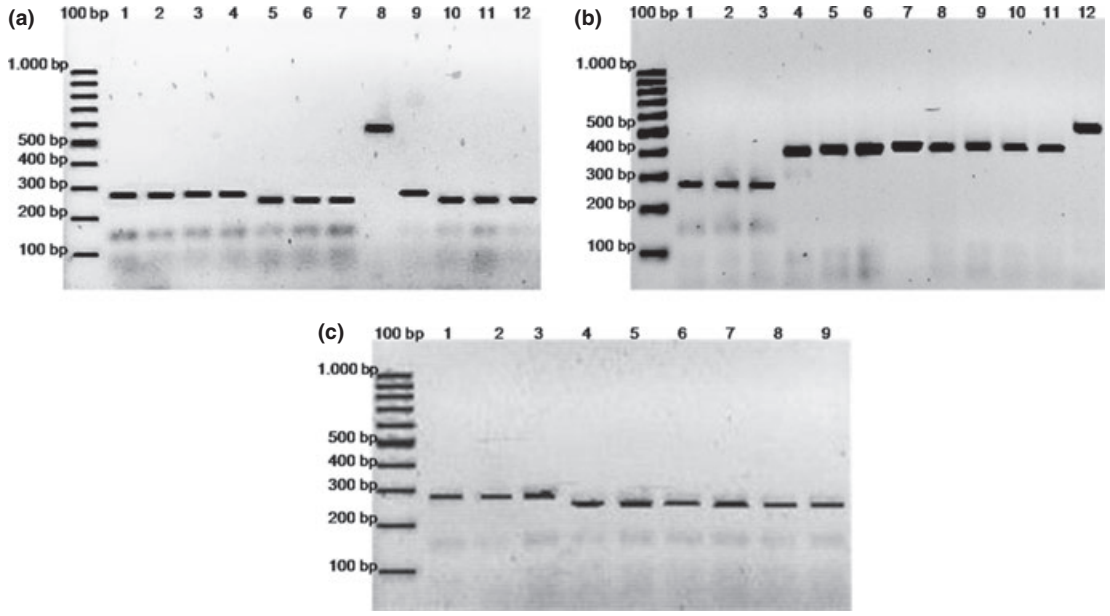

Figure 2 Agarose gel (2.5%) showing restriction fragment length polymorphism (RFLP) based on digestion of 16S rDNA fragments with the enzyme *Hae*III in species of the genus *Crassostrea* from the estuarine-lagoon region of Cananéia.

Table 4 Fragment sizes (bp) generated by *Hae*III for *C. brasiliana*., *C. rhizophorae*., *Crassostrea* sp. and *C. gigas*

	C. brasiliana **(JN849099)**	***C. rhizophorae*** **(JN849104)**	***Crassostrea*** **sp. (JN849108)**	***C. gigas*** **(DQ839414.1)**
Fragment 1	37 bp	38 bp	36 bp	59 bp
Fragment 2	55 bp	55 bp	55 bp	55 bp
Fragment 3	24 bp	261 bp	391 bp	374 bp
Fragment 4	235 bp	131 bp		
Fragment 5	131 bp			

the largest fragments generated are 422, 216 and 120 bp for *C. rhizophorae*, *C. brasiliana* and *Crassostrea* sp. respectively.

Once the largest fragment generated by *Hae*III has 391 bp for *Crassostrea* sp. and 374 bp for *C. gigas*, *Alu*I and *Mse*I enzymes can be used to discriminate these species in localities where *C. gigas* occurs (Southern coast). Among the enzymes that generate unique cuts for each one of them, we can cite *Ban*II and *Psi*I for *Crassostrea* sp. and *Bsw*I for C. gigas (Fig. 3).

Molecular phenetic analysis

Samples of *C. brasiliana* specimens, from four locations in the Cananéia estuary (Cb04, Cb128, Cb190 and Cb232) and one from the Guaraú River (Cb281) were identified through RFLP and sequenced for comparison (accession numbers JN849099–JN849103). Sequences of *C. rhizophorae* were obtained from individuals from four localities (Cr26, Cr67, Cr100 and Cr142) (accession numbers JN849104–JN849107). Sequences of *Crassostrea* sp. were obtained from spat collectors (CspS01, Csp03, Csp09, Csp10 and Csp15) (accession numbers JN849108–JN849112). The neighbour-joining dendrogram (Saitou & Nei 1987), obtained from the K2P distance (Kimura 1980) shows the genetic distances and clustering of related species (Fig. 4). The bootstrap values indicate the branches' consistency index. The values obtained were high, around 100, showing the branches robustness.

C. virginica (Gmelin) and *C. corteziensis* (Hertlein) occupied an intermediate position between *C. brasiliana* and *C. rhizophorae*, indicating that although

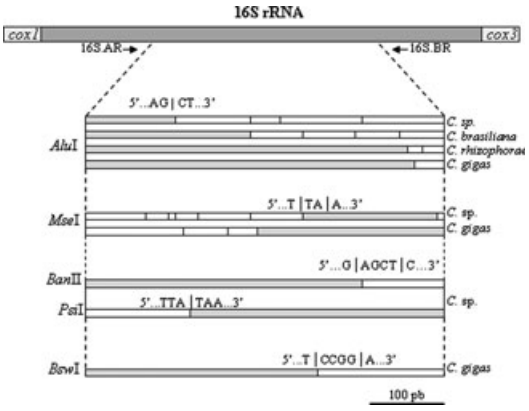

Figure 3 Schematic representation of the restriction sites of *Alu*I, *Mse*I, *Ban*II, *Psi*I and *Brw*I analysed *in silico*.

these two species coinhabit the same estuary, they are at a genetic distance larger than that of other American species that do not occur along the Atlantic coast of Brazil. According to the K2P matrix, the genetic distance between *C. brasiliana* and *C. rhizophorae* is 10.2%. *C. rhizophorae* is genetically closer to *C. virginica* (8.9%), which is distributed from the Gulf of Mexico to the Atlantic coast of North America, than to *C. brasiliana*. The latter, in turn, is closer to *C. corteziensis* (4.4%), which occurs in mangrove areas being distributed from the Gulf of California to northern Peru (Páez-Osuna & Marmolejo-Rivas 1990). The genetic distance between *C. gigas* and *C. brasiliana* is 16.5%, whereas that between *C. gigas* and *C. rhizophorae* is 13.9% (Table 5).

The juveniles obtained from spat collectors grouped with the species from Pacific and Indian Oceans (Fig. 4). The sequencing of these specimens was identical to an oyster species recorded in Pará, north of Brazil (EF473280.1) and they grouped with sequences of an oyster from Beihai, Guangxi Province, China (HQ6609 84.1).

The genetic distance of oysters obtained from artificial collectors was 17.8% in relation to *C. brasiliana*, 17.6% for *C. rhizophorae* and 10.3% for *C. gigas*, showing high genetic divergence from these species (Table 5). Juveniles' genetic distance from the sequence of specimens from China was only 0.3%.

The 16S rRNA sequence comparison clearly shows the difference between the three species found in the estuary, demonstrating that the PCR-RFLP diagnostic method is efficient in consistently identifying the species studied.

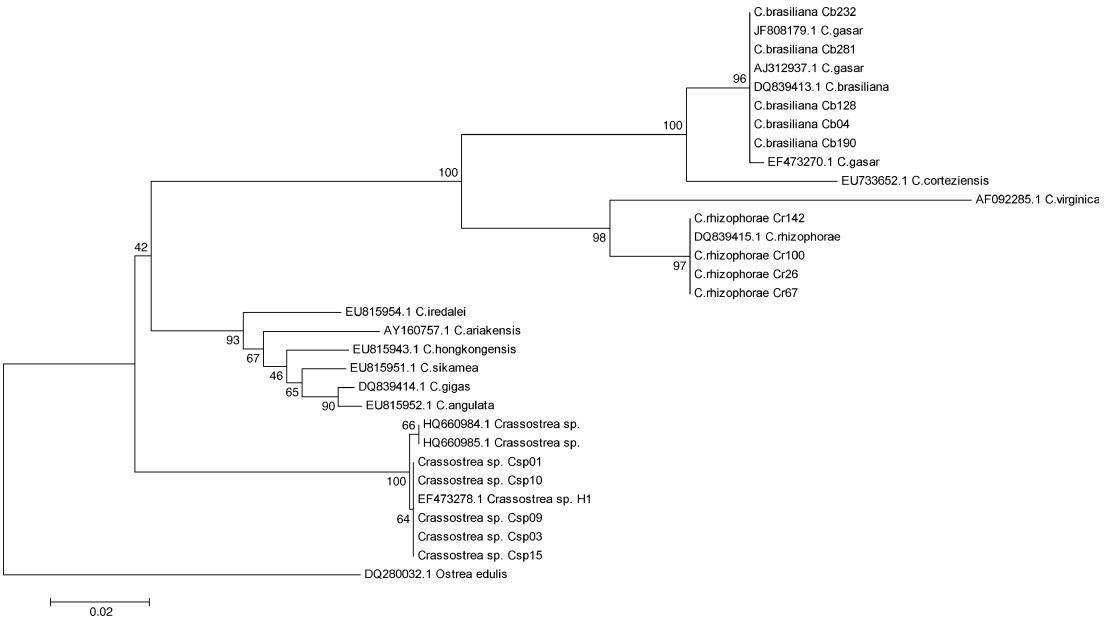

Figure 4 Neighbour-joining dendrogram of the 16S rDNA fragment of oysters of the genus *Crassostrea* collected in the Cananéia and Peruíbe estuary regions and obtained from GenBank, constructed from the Kimura two-parameter distances. The bootstrap values are indicated on the branches.

Table 5 Distance matrix (K2P) of the 16S rDNA fragment between oysters of the genus *Crassostrea*

		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
1	DQ839413.1_Cbrasiliana	0.000																			
2	Cb04, Cb128, C190, Cb232, Cb281	0.000	0.000																		
3	AJ312937.1_C. gasar	0.000	0.000	0.000																	
4	JF808179.1_C. gasarr	0.000	0.000	0.000	0.000																
5	EF473270.1_C. gasar	0.003	0.003	0.003	0.003	0.000															
6	EU733652.1_C_corteziensis	0.044	0.044	0.044	0.044	0.041	0.000														
7	AF092285.1_C_virginica	0.174	0.174	0.174	0.174	0.178	0.195	0.000													
8	DQ839415.1_C_rhizophorae	0.102	0.102	0.102	0.102	0.106	0.112	0.089	0.000												
9	2P29, 3P20, 4P29, 5P24	0.102	0.102	0.102	0.102	0.106	0.112	0.089	0.000	0.000											
10	DQ839414.1_C. gigas	0.165	0.165	0.165	0.165	0.152	0.189	0.185	0.139	0.139	0.000										
11	EU815951.1_C. sikamea	0.158	0.158	0.158	0.158	0.162	0.186	0.185	0.143	0.143	0.021	0.000									
12	EU815952.1_C. angulata	0.168	0.168	0.168	0.168	0.172	0.193	0.189	0.143	0.143	0.008	0.010	0.000								
13	AY160757.1_C. ariakensis	0.171	0.171	0.171	0.171	0.175	0.196	0.206	0.162	0.162	0.046	0.041	0.044	0.000							
14	EU815943.1_C. hongkongensis	0.161	0.161	0.161	0.161	0.165	0.185	0.192	0.149	0.149	0.027	0.027	0.024	0.035	0.000						
15	EU815954.1_C. iredalei	0.155	0.155	0.155	0.155	0.158	0.182	0.206	0.159	0.159	0.046	0.041	0.044	0.049	0.035	0.000					
16	HQ660984.1_Crassostrea_sp.	0.181	0.181	0.181	0.181	0.185	0.195	0.217	0.172	0.172	0.106	0.109	0.106	0.099	0.103	0.096	0.000				
17	HQ660985.1_Crassostrea_sp.	0.181	0.181	0.181	0.181	0.185	0.195	0.217	0.172	0.172	0.106	0.109	0.106	0.099	0.103	0.096	0.000	0.000			
18	EF473280.1_Crassostrea_sp.	0.178	0.178	0.178	0.178	0.181	0.199	0.220	0.176	0.176	0.103	0.106	0.103	0.096	0.099	0.093	0.003	0.003	0.000		
19	Csp01, Csp03, Csp09, Csp10, Csp15	0.178	0.178	0.178	0.178	0.181	0.199	0.220	0.176	0.176	0.103	0.106	0.103	0.096	0.099	0.093	0.003	0.003	0.000	0.000	
20	DQ280032.1_O_edulis	0.230	0.230	0.230	0.230	0.230	0.230	0.230	0.262	0.198	0.198	0.152	0.139	0.152	0.139	0.149	0.152	0.155	0.155	0.155	0.000

The grey shades were used to highlight the genetic distances among species.

Discussion

The difficulty in accurately identifying oyster species through morphological characteristics is due to their greater variation in shell morphology than most bivalve molluscs and the wide range of tolerance to environmental parameters. Due to the high phenotypic plasticity of oysters, morphological analysis is of little value for specimen identification and for the taxonomy as a whole (Boudry, Heurtebise & Lapègue 2003).

It was observed that oysters in the subtidal zone (ST) had thinner, flatter and more regular valves. Intertidal oysters (IT) had thicker and more rigid valves and were quite irregular. PIT oysters gathered on mangrove roots, forming small clusters, had a whitish colour and were easily distinguishable from other mangrove oysters. On the other hand, cultivation oysters grew more homogeneously than those collected from the natural environment.

When collected, the subtidal oysters (ST) were clean, with virtually no encrustations. Intertidal oysters (IT) attached on mangrove roots have many encrustations, such as barnacles (balanoids) and other molluscs, and were attached to each other.

The PCR-RFLP analysis with the use of the *Hae*III endonuclease was effective in identifying the oyster species in Cananéia, as observed by other authors in different regions of the Brazilian coast (Pie *et al.* 2006; Melo *et al.* 2010a).

With the species identification, it was possible to determine their spatial distribution in the Cananéia estuary. *C. brasiliana* occurred both in the rocky subtidal zone (ST) and on mangrove roots in the intertidal zone (IT), whereas *C. rhizophorae* was only found on the intertidal zone (IT) and had no presence in areas with very low salinity (0–10 g L^{-1}). On mangrove roots, where both species have occurred, the proportion of *C. rhizophorae* is the same of *C. brasiliana* as demonstrated using χ^2 test. These data show that *C. brasiliana* has wider distribution in the estuary and tolerates a wider range of salinity.

The χ^2 test also showed that the proportion of *C. rhizophorae* in the PIT group was greater than *C. brasiliana,* demonstrating that the popular knowledge of local fisherman is helpful to identify these species in the estuary.

Concerning to biometric data, *C. brasiliana* were significantly larger than *C. rhizophorae*, indicating that this species probably has higher growth rate than *C. rhizophorae* (Table 3).

Pereira *et al.* (2003) evaluated the growth of oysters from the *Crassostrea* genus at two sites of the Cardoso Island Ecological Reserve located at Cananéia estuary. Oysters attached on mangrove roots of different sizes were marked and numbered with a total of 140 individuals and their growth was monitored monthly. The authors identified the existence of two batches, one of slow growth that reaches the minimum commercial size (50 mm) in around 28 months, representing 72% of the oysters studied, and a fast-growing batch (28%), reaching commercial size in around 19 months. The slow-growing batch probably corresponds to *C. rhizophorae* and the fast-growing one to *C. brasiliana*, as observed in this study. In lagoon estuarine system of Cananéia, Iguape and Paranaguá, the extractive community has empirically named slow-growth oysters 'parangas', which this study confirmed as belonging to the *C. rhizophorae* species ($P < 0.0001$).

Although we have demonstrated that in the Cananéia region the *C. rhizophorae* showed decreased growth compared to *C. brasiliana,* in other countries, such as Colombia, Cuba and Venezuela, it has been reported that *C. rhizophorae* from seed capture reaches commercial size between 50 and 80 mm after 4–8 months of culture (Wedler 1980; Rodríguez & Frías 1992; Buitrago, Buitrago, Fretes & Lodeiros 2009). In Cananéia, Pereira and Chagas Soares (1996) observed that oysters obtained from spat collectors can reach commercial size (>50 mm) after 17 months of cultivation, although the authors were not aware of the taxonomic nomenclature presently known.

Based on these data, we demonstrate that it is not possible to infer that the greater or lesser growth observed for each species is due to taxonomic differences. Size differences can result from abiotic conditions more favourable to one species than to the other. However, specifically for the Cananéia region, this can be used as a phenotypic characteristic to distinguish the local species.

For *C. brasiliana*, ST oysters were significantly larger than IT oysters with respect to all measurements taken. As they are constantly submerged, ST oysters suffer less extraction pressure by fishermen and can feed continuously. Moreover, populations attached on mangrove roots are exposed to open-air and weather conditions during the

normal high and low tide cycles. This is when they close their valves and stop filtering their food, which can cause lower growth rate. On the other hand, these oysters are more resilient and survive several days out of water, a highly desirable feature for local fishermen who live on the extraction and commercialization of such oysters.

It is noteworthy that the oyster cultivation in Cananéia area is an intermediate activity between extraction and integral cultivation in which oysters are taken from mangroves with the minimum length allowed by environmental legislation (50 mm) and placed in trays for a 4–6 month grow-out period until they reach more attractive commercial sizes (between 70 and 100 mm). Certainly, the oyster producers in the region capture oysters from mangroves with a greater growth potential than those commonly known as 'parangas' (*C. rhizophorae*), once all oyster samples from cultivation belongs to *C. brasiliana*. This selection by producers may compromise the natural stock of one species in favour of another that ends up being less exploited.

Oysters originating from spat collection in the natural environment showed the same banding pattern of *C. gigas* in the *Hae*III-based RFLP analysis (Pie *et al.* 2006; Melo *et al.* 2010b). However, when analysing the 16S rRNA sequences, these oysters grouped with Pacific and Indian Oceans species, but showed some genetic distance from *C. gigas* (10.3%) (Table 4). According to the results generated here from the *in silico* analysis of 16S rRNA sequences of *Crassostrea* sp. (Indo-Pacific origin) and *C. gigas*, the *Hae*III was not effective in discriminating between these two species. Thus, we suggest the use of other enzymes, such as *Alu*I and *Mse*I (Fig. 3) in localities where *C. gigas* occurs like in Southern coast of Brazil, where Melo *et al.* (2010a) found the bioinvasion of the Japanese oyster *C. gigas* in natural beds of native species in Florianopolis, Santa Catarina State. The enzymes that generate unique cuts (Fig. 3) may also be useful to discriminate these species.

In the phenetic analysis, sequences of *Crassostrea* sp. showed no differences from those collected by Varela *et al.* (2007) and they clustered with sequences of an oyster from China Sea (Liu *et al.* 2011) (Table 5 and Fig. 4). Therefore, we suggest this is the first record of this Indo-Pacific species in the estuarine region of Cananéia (25°00′S), State of São Paulo.

This study used DNA sequences from GenBank. No information was found about a formal description of this unknown species and it was identified only to genus level. Comparing with some reliable sequences in the databases, genetic distance among Brazilian and Chinese sequences was only 0.3% (Table 5). According to Liu *et al.* (2011), the average K2P distances within and between oysters species for 16S rDNA varied from 0% to 2.0% and 0.5% to 23,3% respectively. Probably, these specimens belong to the same species.

As *Crassostrea* sp. was observed both in Pará and Cananéia (north and southeast of Brazil respectively), it probably occurs along the Brazilian coast, although it has not yet been detected in studies carried out elsewhere in Brazil (Ignacio *et al.* 2000; Lapègue *et al.* 2002; Pie *et al.* 2006; Melo *et al.* 2010a,b; Lazoski *et al.* 2011; Lazoski *et al.* 2011). Thus, *Crassostrea* sp. may consist in a bioinvasive species with high dispersion power and even being more aggressive than *C. gigas* that is limited to Southern coast.

In this study, the Indo-Pacific species was not observed on mangrove roots or rocky substrates, where oysters larger than 50 mm were collected. Possibly this oyster has slow growth rate and does not reach sizes larger than 50 mm or that breeding banks are located in other areas. Thus, it is suggested new studies to detect breeding banks including sampling of specimens with sizes below 50 mm attached on different substrates in the estuary.

In Brazil, several studies have investigated oysters cultivation based on the capture of seeds in the natural oyster banks (Pereira & Chagas Soares 1996; Alvarenga & Nalesso 2006; Nalesso, Paresque, Piumbini, Tonini, Almeida & Nickel 2008). However, productivity increment of this farming system is related to the correct taxonomic identification to raise the most appropriate fast growing oyster species.

The Lagoon Estuarine system of Cananéia, Iguape and Paranaguá is well conserved from the point of view of human interference therefore it is important to carry out spat collection surveys at different locations and seasons to investigate the occurrence of larvae and seeds from other oyster species in the region. On the other hand, the development of artificial reproduction in hatchery systems, such as that implemented in the State of Santa Catarina in Southern Brazil (Maccachero, Guzenski & Ferreira 2005; Maccacchero, Ferreira

& Guzenski 2007) can be established for further protection of the natural oyster banks and improvement of the oyster farming productivity.

The record of an exotic species was unexpected, as Cananéia-Iguape-Paranaguá estuarine complex is an environmental protection area. Its presence in the estuary, which may have been introduced by ship's ballast water (Loebmann, Mai & Lee 2010), can have strong ecological and environmental implications and could jeopardize the natural stocks of native species. Non-native species are a primary threat to biodiversity. They can cause impacts ranging from the suppression of native populations to changes in ecosystem properties and hybridization with native species (Wonham & Contributers 2006). Direct effects of introducing exotic species result from their immediate interaction with other species through predation, competition, parasitism or disease.

The adoption of new guidelines for farming, sustainable management and conservation of species should be considered. With oyster species distinction in the region, it will be possible to promote a balanced practise, avoiding declines in biomass, with consequent ecological, economic and social impacts. Thus, it will be possible to protect the livelihood of fishermen for several generations.

Acknowledgments

We would like to thank the Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP # 07/50094-0) for granting funds to support this project. Also, we thank Dr. Marcelo Barbosa Henriques for his help with sampling and fieldwork logistic, Dr. Naoyo Yamanaka for the valuable suggestions and manuscript revision. Messrs. Onésio Veríssimo and Antônio Pires for the help during the oysters sampling and spat collection. Dra. Celina Maria Marcondes Pimentel and Ms. Delcira de Fátima Santos Vieira for their assistance during the oyster processing and Daniella Albuquerque de Sousa for the bench help with the PCR-RFLP analysis. This work was developed as part of the full requirements for the PhD's Thesis in Biotechnology at the UMC/FAEP.

References

Akaboshi S. & Pereira O.M. (1981) Ostreicultura na região lagunar-estuarina de Cananéia, São Paulo, Brasil. I. Captação de larvas de ostra Crassostrea brasiliana (Lamarck, 1819) em ambiente natural. *Boletim do Instituto de Pesca* **8**, 87–104.

Alvarenga L. & Nalesso R.C. (2006) Preliminary assessment of the potential for mangrove oyster cultivation in Piraquê-açu river estuary (Aracruz, ES). *Brazilian Archives of Biology and Technology* **49**, 163–169.

Boudry P., Heurtebise S., Collet B., Cornette F. & Gérard A. (1998) Differentiation between populations of the Portuguese oyster, *Crassostrea angulata* (Lamark) and the Pacific oyster, *Crassostrea gigas* (Thunberg), revealed by mtDNA RFLP analysis. *Journal of Experimental Marine Biology and Ecology* **226**, 279–291.

Boudry P., Heurtebise S. & 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.

Buitrago E., Buitrago J., Fretes L. & Lodeiros C. (2009) Identificación de factores que afectan al crecimiento y la supervivencia de la ostra de mangle, *Crassostrea rhizophorae* (Guilding, 1828), bajo condiciones de cultivo suspendido en la laguna de La Restinga, Isla de Margarita, Venezuela. *Zootecnia Tropical* **27**, 79–90.

Galtsoff P.S. (1964) The American Oyster *Crassostrea virginica* Gmelin. *Fishery Bulletin of the Fish and Wildlife Service* **64**, 453.

Giribet G., Okusu A., Lindgren A.R., Huff S.W., Schrodl M. & Nishiguchi M.K. (2006) Evidence for a clade composed of molluscs with serially repeated structures: monoplacophorans are related to chitons. *Proceedings of the National Academy of Sciences of the United States of America* **103**, 7723–7728.

Ignacio B.L., Absher T.M., Lazoski C. & Solé-Cava A.M. (2000) Genetic evidence of the presence of two species of *Crassostrea* (Bivalvia: Ostreidae) on the coast of Brazil. *Marine Biology* **136**, 987–991.

Kimura M. (1980) A simple method for estimating evolutionary rate of base substitutions through comparative studies of nucleotide sequences. *Journal of Molecular Evolution* **16**, 111–120.

Klinbunga S., Khamnamtong N., Puanglarp N., Jarayabhand P., Yoosukh W. & Menasveta P. (2005) Molecular taxonomy of cupped oysters (*Crassostrea, Saccostrea,* and *Striostrea*) in Thailand based on COI, 16S, and 18S rDNA polymorphism. *Marine Biotechnology* **7**, 306–317.

Lam K. & Morton B. (2006) Morphological and mitochondrial-DNA analysis of the indo-west pacific rock oysters (Ostreidae: *Saccostrea* species). *Journal of Molluscan Studies* **72**, 235–245.

Lapègue S., Boutet I., Leitão A., Heurtebise S., Garcia P., Thiriot-Quiévreux C. & Boudry P. (2002) Trans-Atlantic distribution of a mangrove oyster species revealed by 16S mtDNA and karyological analyses. *Biological Bulletin* **202**, 232–242.

Lazoski C., Gusmão J., Boudry P. & Solé-Cava A.M. (2011) Phylogeny and phylogeography of Atlantic oys-

ter species: evolutionary history, limited genetic connectivity and isolation by distance. *Marine Ecology Progress Series* **426**, 197–212.

Liu J., Li Q., Kong L., Yu H. & Zheng X. (2011) Identifying the true oysters (Bivalvia: Ostreidae) with mitochondrial phylogeny and distance-based DNA barcoding. *Molecular Ecology Resources* **11**, 820–830.

Loebmann D., Mai A.C.G. & Lee J.T. (2010) The invasion of five alien species in the Delta do Parnaíba Environmental Protection Area, Northeastern Brazil. *Revista de Biología Tropical* **58**, 909–923.

Maccacchero G.B., Ferreira J.F. & Guzenski J. (2007) Influence of stocking density and culture management on growth and mortality of the mangrove native oyster *Crassostrea* sp. in southern Brazil. *Biotemas* **20**, 47–53.

Maccachero G.B., Guzenski J. & Ferreira J.F. (2005) Allometric growth on mangrove oyster *Crassostrea rhizophorae* (Guilding, 1828), cultured in Southern Brazil. *Revista Ciência Agronômica* **36**, 400–403.

McCarthy K.R., Brown R.F., Johnstone M.B. & Wheeler A.P. (1998) Phylogenetic comparison of the family Bivalvia using the 16S-rRNA gene in correlation with shell microstructure (unpublished). Available at http://www.ncbi.nlm.nih.gov/nuccore/ (accessed 18 August 2011).

Melo C.M.R., Silva F.C., Gomes C.H.A.M., Solé-Cava A.M. & Lazoski C. (2010a) *Crassostrea gigas* in natural oyster banks in southern Brazil. *Biology Invasions* **12**, 441–449.

Melo A.G.C., Varela E.S.V., Beasley C.R., Schneider H., Sampaio I., Gaffney P.M., Reece K.S. & Tagliaro C.H. (2010b) Molecular identification, phylogeny and geographic distribution of Brazilian mangrove oysters (Crassostrea). *Genetics and Molecular Biology* **33**, 564–572.

Nalesso R.C., Paresque K., Piumbini P.P., Tonini J.F.R., Almeida L.G. & Nickel V.M. (2008) Oyster spat recruitment in Espírito Santo State, Brazil, using recycled material. *Brazilian Journal of Oceanography* **56**, 281–288.

Nei M. & Kumar S. (2000) *Molecular Evolution and Phylogenetics*. Oxford University Press, New York, 333pp.

Páez-Osuna F. & Marmolejo-Rivas C. (1990) Trace metals in tropical coastal lagoon bivalves, *Crassostrea cortezienensis*. *Bulletin of Environmental Contamination and Toxicology* **45**, 538–544.

Palumbi S., Martin A., Romano S., Mcmilan W.O., Stice L. & Grabowski G. (1991) *The Simple Fool's Guide to PCR*. Department of Zoology and Kewalo Marine Laboratory, University of Hawaii, Honolulu, Hawaii.

Pereira O.M. & Chagas Soares F. (1996) Análise da criação de ostra *Crassostrea brasiliana* (LAMARCK, 1819), no sítio Guarapari, na região Lagunar-Estuarina de Cananéia-SP. *Boletim do Instituto de Pesca* **23**, 135–142.

Pereira O.M., Henriques M.B. & Machado I.C. (2003) Estimativa da curva de crescimento da ostra *Crassostrea brasiliana* em bosques de mangue e proposta para sua extração ordenada no estuário de Cananéia, SP, Brasil. *Boletim do Instituto de Pesca* **29**, 19–28.

Perez-Enriquez R., Ibarra S. & Ávila A.M. (2008) Population genetics of the oyster *Crassostrea corteziensis* in the Gulf of California. *Ciencias Marinas* **34**, 479–490.

Pie M.R., Ribeiro R.O., Boeger W.A., Ostrensky A., Falleiros R.M. & Angelo L. (2006) 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. *Aquaculture Research* **37**, 1598–1600.

Reece K.S., Cordes J.F., Stubbs J.B., Hudson K.L. & Francis E.A. (2008) Molecular phylogenies help resolve taxonomic confusion with Asian *Crassostrea* oyster species. *Molecular Biology* **153**, 709–721.

Rios E.C. (1994) *Seashells of Brazil*. Editora Fundação Universidade de Rio Grande (FURG), Rio Grande, RS, 481pp.

Rodríguez J. & Frías J.A. (1992) Tropical mangrove oyster production from hatchery-raised seed in Cuba. *Journal of Shellfish Research*, **11**, 455–460.

Rosenberg G. (2009) *Malacolog Version 4.1.1. A Database of Western Atlantic Marine Mollusca*. The Academy of Natural Sciences. [www database (version 4.1.1)]. Available at http://www.malacolog.org.

Saitou N. & Nei M. (1987) The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Molecular Biology and Evolution* **4**, 406–425.

Sanchez A., Quinteiro J., Rey-Mendez M., Perez-Martin R. & Sotelo C.G. (2011) Identification and quantification of oyster species larva using real time PCR(unpublished). Available at http://www.ncbi.nlm.nih.gov/nuccore/.

Schwager S.J., Mutchler M.A., Federer W.T. & Scully B.T. (1993) The effect of linkage on sample size determination for multiple trait selection. *Theoretical and Applied Genetics* **86**, 964–974.

Tamura K., Dudley J., Nei M. & Kumar S. (2007) *MEGA4*: molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. *Molecular Biology and Evolution* **24**, 1596–1599.

Thorpe J.P. & Solé-Cava A.M. (1994) The use of allozyme eletrophoresis in invertebrate systematics. *Zoologica Scripta* **23**, 3–18.

UNESCO (1999) *United Nations Educational, Scientific and Cultural Organization - World Heritage Nomination*. IUCN Technical Evaluation Atlantic Forests (southeast) Brazil, Marrakesh, Morocco, 8pp.

Unesco (2005) *United Nations Educational, Scientific and Cultural Organization–World Network of Biosphere Reserves*. SC/EES, Marrakesh, Morocco. The MAB Program, 19pp.

Varela E.S., Beasley C.R., Schneider H., Sampaio I., Marques-Silva N.S. & Tagliaro C.H. (2007) Molecular phylogeny of mangrove oysters (*Crassostrea*) from Brazil.

Journal of Molluscan Studies Advance Access **73**, 229–234.

Vincze T., Posfai J. & Roberts R.J. (2003) NEBcutter: a program to cleave DNA with restriction enzymes. *Nucleic Acids Research* **31**, 3688–3691.

Wang Y., Guofan Z., Liu X. & Guo X. (2008) Classification of common oysters from north China. *Journal of Shellfish Research* **27**, 495–503.

Wedler E. (1980) Experimental spat collecting and growing of the oyster, *Crassostrea rhizophorae* Guilding, in the Ciénaga Grande de Santa Marta, Colombia. *Aquaculture* **21**, 251–259.

Wonham M. (2006) Species invasions. In: *Principle of Conservation Biology* (ed. by M.J. Groon, G.K. Meffe & C.R. Carsol & Contributers), pp. 293–321. Sinauer Associates, Sunderland, MA, USA.

Xia J. & Yu Z. (2008) Identification and distribution of oyster species from the South China Sea (unpublished). Available at http://www.ncbi.nlm.nih.gov/nuccore/197318696.

Xia J., Yu Z. & Kong X. (2009) Identification of seven *Crassostrea* oysters from the South China Sea using PCR–RFLP analysis. *Journal of Molluscan Studies* **75**, 139–146.

Zar J.H. (1996) *Bioestatistical Analysis* (3rd edn). Prentice Hall, New Jersey, 662pp.