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MOLECULAR IDENTIFICATION OF CRYPTIC SPECIES OF OYSTERS (GENUS *Crassostrea* SACCO, 1897) IN THE NORTHEAST ATLANTIC COAST OF BRAZIL

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ABSTRACT

Oysters of the genus *Crassostrea* Sacco, 1897 are widely distributed worldwide, being important extractive and cultivation resources in Brazil. Because they have high phenotypical plasticity and congeneric similarity, identifications based on shell morphology are not always safe. The goal of this study was to identify the oysters of the Bahia State, northeast Brazil, using the molecular tools Polymerase Chain Reaction, Restriction Fragment Length Polymorphism, DNA sequencing and phylogenetic analysis. Oysters were collected at 12 sampling stations, from October 2014 to March 2015 and included samples of rhizomes (aerial roots)/stems of the red mangrove *Rhizophorae mangle* L. and in the sediment near to the underground roots of this one, on berths, natural rock outcrops near the mangrove swamp and in three oyster crops. It was confirmed the presence of two species of oysters: *Crassostrea rhizophorae* (Guilding, 1828) and *C. gasar* (Deshayes, 1830) and that the latter was genetically identical to *C. brasiliana* reported in previous studies on the Brazilian coast. There was no co-occurrence of the two species on the same substrate, but these were found in nearby environments at two sampling points. *Crassostrea rhizophorae* was observed on the rhizomes/stems of *R. mangle*, as well as on artificial concrete walls (berths). The semi-buried oysters near *R. mangle*'s subterranean roots and adhered to small rocks of a rocky outcrop were *C.gasar*, which was also the exclusive oyster of the crops.

Key words: biodiversity; bivalves; genetic identification; marine resources; ostreiculture.

IDENTIFICAÇÃO MOLECULAR DE ESPÉCIES CRÍPTICAS DE OSTRAS (GÊNERO *Crassostrea* SACCO, 1897) NA COSTA ATLÂNTICA NORDESTE DO BRASIL

RESUMO

As ostras do gênero *Crassostrea* Sacco, 1897 são amplamente distribuídas mundialmente, sendo importantes recursos extrativistas e de cultivo no Brasil. Por possuírem alta plasticidade fenotípica e semelhança congenérica, as identificações baseadas na morfologia da concha nem sempre são seguras. O objetivo deste estudo foi identificar as ostras do estado da Bahia, nordeste do Brasil, utilizando as ferramentas moleculares Reação em Cadeia da Polimerase, Polimorfismo do Comprimento de Fragmentos de Restrição, sequenciamento de DNA e análise filogenética. As ostras foram coletadas em 12 estações amostrais, de outubro de 2014 a março de 2015 e incluíram coletas sobre rizomas (raízes aéreas)/caules do mangue vermelho *Rhizophorae mangle* L. e no sedimento próximo às raízes subterrâneas deste, em atracadouros, afloramentos rochosos naturais próximos ao manguezal e em três cultivos de ostras. Confirmou-se a presença de duas espécies de ostras: *Crassostrea rhizophorae* (Guilding, 1828) e *C. gasar* (Deshayes, 1830) e que esta última foi geneticamente idêntica à *C. brasiliana*, relatada em estudos anteriores na costa brasileira. Não houve co-ocorrência das duas espécies no mesmo substrato, mas em dois pontos amostrais estas foram encontradas em ambientes próximos. *Crassostrea rhizophorae* foi observada nos rizomas/caules de *R. mangle*, bem como em paredes artificiais de concreto (atracadouros). As ostras semi-enterradas perto das raízes subterrâneas de *R. mangle* e aderidas a pequenas rochas de um afloramento rochoso foram *C. gasar*, que também foi a ostra exclusiva nos cultivos.

Palavras-chave: biodiversidade; bivalves; identificação genética; recursos marinhos; ostreicultura.

INTRODUCTION

Oysters (Bivalvia: Ostreidae) are mollusks of economic interest with great worldwide occurrence, distributed between latitudes 64°N and 44°S. Species of the genus *Crassostrea* Sacco, 1897 inhabit mainly estuarine regions and present a shell with a generally elongated shape and with unequal valves: the upper (right) is smaller and flatter, and the lower (left), by which the oyster attaches to the substrate, is larger and concave (Galtsoff, 1964). Oysters of the Brazilian coast belong to the subfamily Ostreinae Rafinesque, 1815 and include representatives of the genus *Lopha*, *Ostrea* and *Crassostrea* (Rios, 2009).

Oysters of the genus *Crassostrea* present high morphological plasticity and phenotypic similarity among species. According to Ignacio et al. (2000), the type of the substrate as well as other environmental factors may determine the shape and appearance of these oysters. This has caused difficulties in the correct identification for several decades, mainly in relation to cryptic species, culminating in erroneous identifications and several cases of synonyms, a fact that has also occurred in Brazil. In recent years, with the advent of molecular tools, identifications have become more secure.

The safe identification of oyster populations has great practical value in the cultivation of these organisms and also as a subsidy for the preservation and management of natural stocks. Oysters *Crassostrea* spp. are cultivated in several places of the Brazilian coast, as in the states of Santa Catarina, São Paulo, Rio de Janeiro, Espírito Santo, Bahia, Sergipe, Rio Grande do Norte and Pará (IBGE, 2016). The coast of Bahia State, which is about 1,100 km long, is characterized by extensive beaches and several estuaries and bays. Regarding estuarine regions, the All Saints' Bay stands out in the central region of the state and the Camamu Bay, further south. Oysters of the genus *Crassostrea* are extractive resources of great socio-economic relevance in the region, mainly for the traditional populations, who use them for their own consumption and exploitation (Lenz and Boehs, 2011). Small scale crops practiced both on boards (tables) and in long line systems are observed in several places, mainly in the All Saints' Bay and in the so-called "South Coast" of the State. One of the difficulties in the region is precisely the correct identification of the species, which would help in obtaining seedlings for local oyster farming, including the production of high quality spats via larviculture. In a more general context, the safe identification of the oyster species is also fundamental to know specific aspects related to parasitism and diseases, accumulation of chemical and microbiological contaminants, physiological responses to impacts of human activities and nutritional quality of each species.

In this study we aim to make, for the first time, the genetic identification of oysters from a representative stretch (about 500 km) on the coast of the Bahia State, northeastern Brazil, in order to generate useful subsidies for oyster farming of the region, as well as, in a broader perspective, to serve as a basis for management programs and conservation of natural stocks.

MATERIAL AND METHODS

Study area, sampling points and collection procedures

Samplings were made between October 2014 and March 2015 at 12 sampling stations (St1-St12) located between latitudes 12º39'27.00"S and 15º42'41.41"S, which were located in the municipalities of Cachoeira (St1), Camamu (St2-St6), Maraú (St7 and St8), Ilhéus (St9 and St10) and Canavieiras (St11 and St12) (Figure 1). The collections were previously authorized by the Chico Mendes Institute for Biodiversity Conservation - ICMBio, Brazil (License number 20912-3), including sampling points located in federal conservation areas [St1 - Extractive Reserve (RESEX) Bay of Iguape; St11 and St12 - RESEX of Canavieiras]. The oysters, with a total of 320 specimens, were collected with the aid of a knife during low tide. The oysters were obtained (i) on aerial roots (rhizomes) and stems of *Rhizophora mangle* (St2 - Ilha Pequena, n = 30; St3 - Cajaíba, n = 30; St4 - Porto do Campo, n = 30; St5 - Ilha do Caranguejo, n = 30; St6 - Ponta de Caieiras, n = 30; St7b – Maraú, n = 10; St8b – Ilha de Tanques, n = 10; St9 - Barra do Itaípe, n = 10), (ii) on artificial concrete structures (berth) (St11 – Canavieiras, n = 30), (iii) on natural rock outcrops (composed of small rocks, approximately with 10 to 30 cm in diameter) and artificial concrete structures (berth) (St10 - Pontal Bay, n = 30), (iv) in sediment near mangrove roots (St12 – Campinhos, n = 30), and (v) on crops (St1 - Bay of Iguape, n = 30; St7a – Maraú, n = 10; St8a - Ilha de Tanques, n = 10) (Figure 1). The specimens were transported to the laboratory in pails containing a small portion of sea water from each location.

Laboratory procedures

The initial processing was done in the Laboratory of Marine Mollusks of the State University of Santa Cruz (LMM / UESC), where the oysters were placed in aquaria and soon after processed. These were measured for height (distance between the umbo and the ventral valve margin) (Galtsoff, 1964), using a digital caliper. The oysters were opened with the aid of a knife/ scalpel and fragments of the adductor muscle were separated from each specimen. The fragments were stored in a freezer at -80°C. The molecular procedures were initially made at the UESC Veterinary Genetics Laboratory. Total DNA extraction was done using the phenol-chloroform protocol (OIE, 2003). The Polymerase Chain Reaction (PCR) was done with a total of 100-200 ng of DNA, 0.25 U of Taq Polymerase (Invitrogen®), 2.5 mM of dNTP, 10.0 pmol of each primer and 2.5 mM of $MgCl_2$. Ultrapure water was used as the negative control and previously sequenced samples were used as positive control. The 16S rDNA primers pair of 16S.AR (5'-CGCCTGTTTATCAAAAACAT-3') and 16S.BR (5'-CCGGTCTGAACTCAGATCACGT-3') (Palumbi et al., 2002) were used for a total of 35 cycles, with annealing temperature of 56°C for 40s, yielding 530 base pairs (bp) amplicons, using Invitrogen's 1 Kb Plus DNA Ladder marker. All samples (n = 320) were also submitted to Reactions of digestion of DNA fragments by restriction enzymes (PCR-RFLP). The product was digested with 10U Hae III endonuclease, with final volume of 10ul. Samples

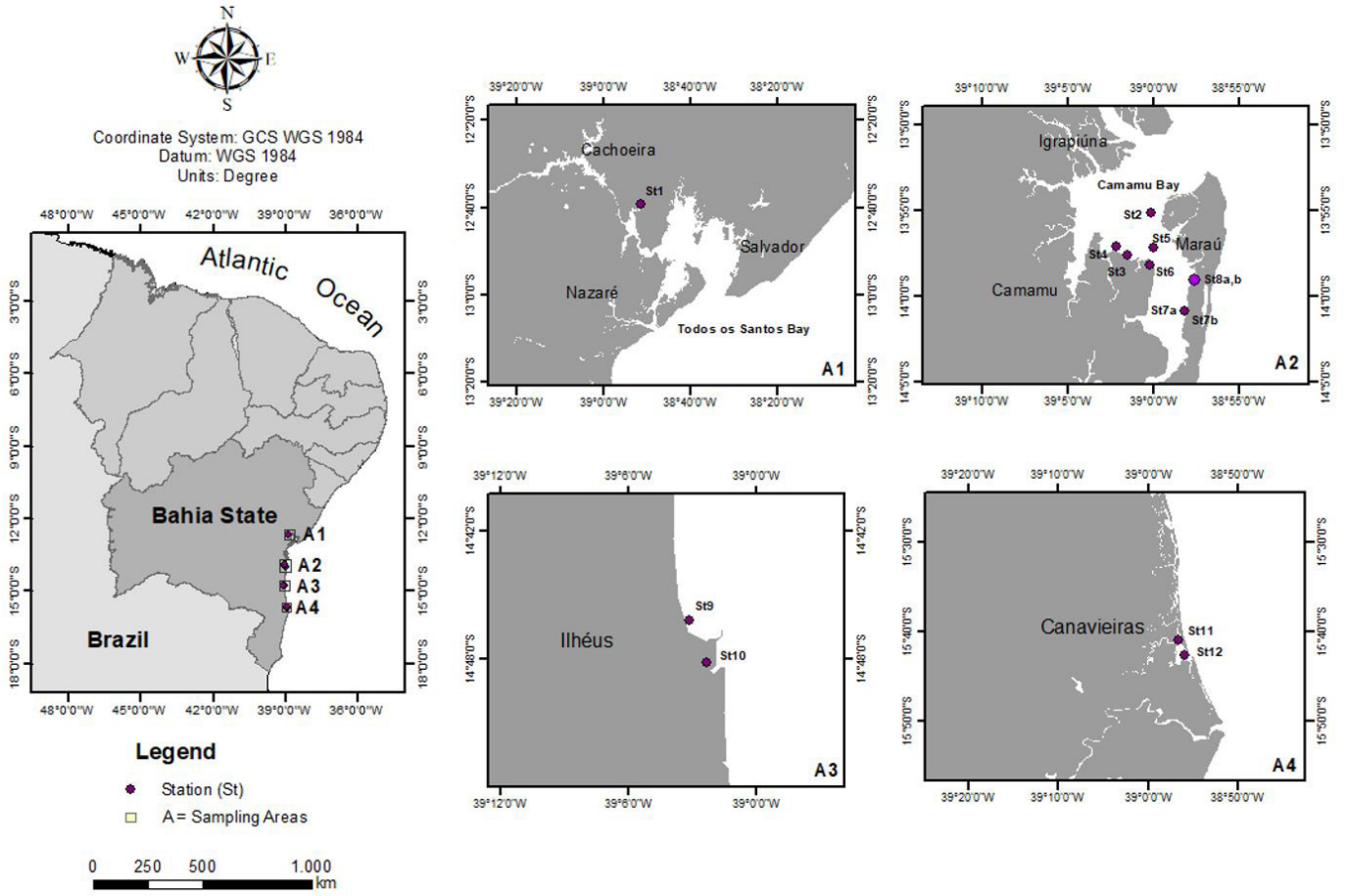

Figure 1. Study area with indication of the sampling areas (A1-A4) and sampling stations (St1-St12) of *Crassostrea* spp. Composition: Ricardo L. Viana.

were incubated for 1 hr at 37°C and subsequently observed on agarose gel 2% stained with Syber Safe®, with amplicons of 230 bp for *C. gasar* and 260 bp for *C. rhizophorae* (Pie et al., 2006). For the bands obtained by PCR-RFLP, was used the Phix 174 marker (Invitrogen). After PCR-RFLP, 20 samples were selected for DNA sequencing, which was done by ACTGene Molecular Analyzes (Center of Biotechnology, Federal University of Rio Grande do Sul, Porto Alegre, RS, Brazil). The company used an AB 3500 automatic sequencer (Genetic Analyzer) armed with 50 cm capillaries and POP7 polymer (Applied Biosystems - AB) for sequencing. DNA-molds (50 ng) were labeled using 2.5 pmol of primer (16S.AR and 16S.BR) and 0.5 µl of BigDye Terminator v3.1 Cycle Sequencing Standard reagent (AB) in one volume end of 10 µL. The labeling reactions were performed on a GeneAmp PCR System 9700 (AB) thermocycler with an initial denaturation step at 96°C for 3 min followed by 25 cycles of 96°C for 10 sec, 55°C for 5 sec and 60°C for 4 min. Once labeled, the samples were purified by precipitation with 75% isopropanol and washing with 60% ethanol. The precipitated products were diluted in 10 µL of Hi-Fi formamide (AB), denatured at 95°C for 5 min, cooled on ice for 5 min and electroinjected into the automated sequencer.

Interpretation of results

The results of DNA sequencing were compared using the Basic Local Alignment Search Tool (BLAST) with other data deposited at the National Center for Biotechnology Information (NCBI). Sequence alignments were produced using the BioEdit (Hall, 1999) and ClustalX (Thompson et al., 1997) programs. The nucleotide frequencies and the transition / trans-version ratio were obtained using Mega 6.6 software (Tamura et al., 2013). In the phylogenetic analysis, Neighbor-Joining (NJ) (Saitou and Nei, 1987) and maximum Parsimony (MP) methods were used. Following procedure adopted by Varela et al. (2007), *Ostrea edulis* (Ostreinae) and *Saccostrea cucullata* (Saccostreinae) were used as outgroups. Sequences were compared to access numbers in GenBank. So, for *C. rhizophorae*[1], *C. gasar*[2] and *C. brasiliana*[3], sequences from samples of the Brazilian coast were used, based on the studies of Lapègue et al. (2002) (AJ312938[1]; AJ312937[2]), Introini et al. (2012) (HQ711626[3]), Pie et al. (2006) (DQ839415[1]; DQ839413[3]), Varela et al. (2007) (EF473270[2]), Melo et al. (2010a) (FJ478030[1]; J478027[3]) and Galvão et al. (2013) (JN849104[1]; JN849107[1]; JN849099[3]; JN849103[3]). The analysis involved 24 nucleotide sequences. Our three sequences were deposited in GenBank under

numbers BankIt2177474-SeqLMM1-MK327809, BankIt2177474-SeqLMM2a-MK327810 and BankIt2177474-SeqLMM2b-MK327811.

RESULTS

The study confirmed the existence of two species of oysters, identified as *Crassostrea rhizophorae* and *C. gasar*. The first was found on stems and rhizomes of *R. mangle* (St2-St6, St7b, St8b and St9) and on artificial substrates (berths) (St10 and St11). Samples collected on natural rock outcrops (St10) and buried in mangrove sediments near roots of *R. mangle* (St12), as well as all the oysters obtained from crops (St1, St7a and St8a) were *C. gasar*. No morphological differences were observed between the shells of the two species. The total sample pool (n = 236) of *C. rhizophorae* consisted of oysters with average height of 4.91, SD ± 1.2 cm and of *C. gasar* (n = 84) with 7.47, SD ± 1.77 cm.

PCR results indicated amplicons with the expected size (530 bp) of the genus *Crassostrea* (Figure 2). PCR-RFLP showed approximately 230 bp for *C. gasar* and 260 bp for *C. rhizophorae* (Figure 3). The results obtained by Neighbor-Joining (NJ) and Maximum Parsimony (MP) methods were similar, so we chose to present only the graph of NJ. Comparing the results with the sequences already deposited in NCBI, similarities ranged from 96 to 99% for *C. rhizophorae* and were 100% for *C. gasar* (Figure 4). Both species showed proximity to *C. virginica*, the North American oyster, mainly *C. rhizophorae*. As observed in the cladogram, there was no genetic difference between *C. gasar* and *C. brasiliana*. The dendogram also shows the most distant kinship with the genera *Saccostrea* and *Ostrea*, which are more basal groups.

DISCUSSION

The study confirmed the existence of two cryptic oyster species of genus *Crassostrea* in the region, both inhabiting estuarine regions and sometimes occurring in the same places (as in St10), but not together. *Crassostrea rhizophorae*, as the vernacular name itself indicates ("mangrove oyster"), was found on the aerial roots (rhizomes) and stems of *R. mangle* and also on artificial concrete walls, where it was unique in relation to *C. gasar*. This last species is also known as the "bottom oyster" (Legat et al., 2017) and was observed adhering to small rocks outcrops, as well as in the unconsolidated substrate among mangrove trees, close to *R. mangle* underground roots, clearly at a lower intertidal level than *C. rhizophorae*. Specific distribution of these two species was also evidenced by Ignacio et al. (2000), Galvão et al. (2013) and Almeida et al. (2014). Galvão et al. (2013) verified co-occurrence of both species on mangrove roots in intertidal zone, but observed that all oysters present in subtidal rocks corresponded to *C. brasiliana* (= *C. gasar)*. Therefore, the present and previous studies indicate a predominance of *C. rhizophorae* at higher intertidal levels and a tendency of *C. gasar* to restrict its habitat to levels closer to the sediment or occupying shallow subtidal areas, such as river beds with presence of consolidated substrate (rocks) (Boehs, personal observation).

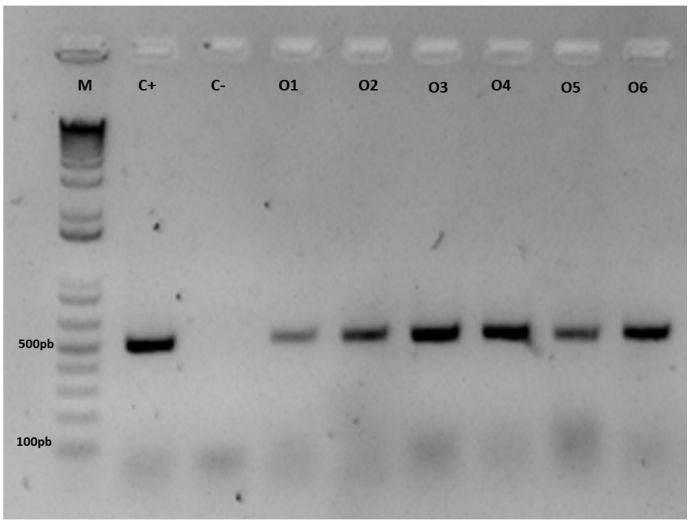

Figure 2. Results of 2% agarose gel electrophoresis with PCR amplifications of the 16S rDNA gene region. The first line shows the molecular marker (M) (1kb Lander – Invitrogen), followed by the positive (C+), negative (C-) control and samples 01-06, all positive (n = 320).

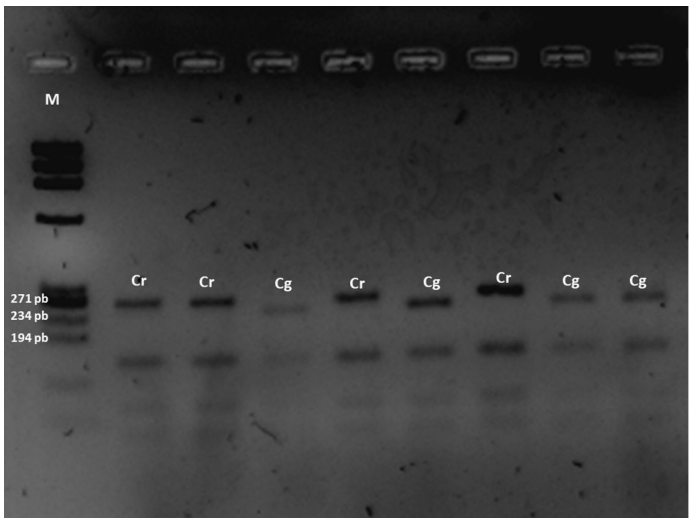

Figure 3. Results of 2% agarose gel electrophoresis with PCR-RFLP amplifications of the 16S rDNA gene region. Cg = *Crassostrea gasar*; Cr = *C. rhizophorae*; M = Molecular marker (Phix 174 (Invitrogen) (n = 320).

With respect to taxonomy, Rios (2009) admits the existence of only one species of the genus *Crassostrea* in Brazilian coast, mentioning that *C. brasiliana* would be in synonymy with *C. rhizophorae*, reporting its distribution from the Caribbean to Uruguay. This synonym was also proposed previously by Singarajah (1980), which also described a new species (*C. paraibanensis*) in the Paraíba River (Paraíba State, northeastern Brazil) based on morphological analysis and physiological characterization.

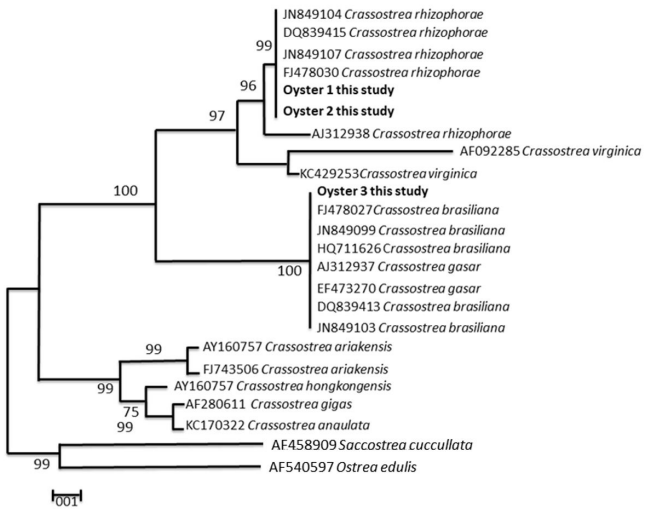

Figure 4. Cladogram of Neighbor-Joining method for genus *Crassostrea* and *Saccostrea cucullata* based on 16S rRNA, using *Ostrea edulis* and *Saccostrea cucullata* as outgroups, with the inclusion of the oysters used in this study, and access numbers in GenBank of species.

Amaral and Simone (2014), through morpho-anatomical studies and from a taxonomic review of oysters of the genus *Crassostrea* occurring from Brazil to the Caribbean, affirm that the species existing in Brazil are *C. brasiliana* (which would occurs from Paraíba State to Santa Catarina State) and *C. mangle* Amaral and Simone, 2014, with occurrence from Pará to Santa Catarina, being the latter equivalent to the known *C. rhizophorae*. These last authors refuted the existence of *C. gasar* in Brazil and affirm that *C. rhizophorae* occurs only in Caribbean.

The results of the present study confirm those obtained in several studies using molecular tools in other localities of the Brazilian coast (Ignacio et al., 2000; Pie et al., 2006; Varela et al., 2007; Melo et al., 2010a; Lazoski et al., 2011; Almeida et al., 2014), which confirmed the existence of two native species of the genus *Crassostrea* in Brazil: *C. rhizophorae* and *C. gasar.* Melo et al. (2010a) sequenced the 16S rRNA gene of oysters from the Paraíba river estuary and concluded that these sequences were identical to those of *C. rhizophorae* published by Varela et al. (2007). So, these authors did not confirm *C. paraibanensis* at this place, as established by Singarajah (1980). In addition to *C. rhizophorae* and *C. gasar*, an unidentified exotic *Crassostrea* species is related for Canela Island, Bragança (Pará State, north of Brazil) (Varela et al., 2007; Melo et al., 2010a) and by Galvão et al. (2013) in Cananéia, São Paulo State, Southeast Brazil. *Crassostrea gigas* (Thunberg, 1793), the "Pacific oyster", also exotic, is cultivated in Santa Catarina, where it meets the appropriate temperature and salinity conditions for its development, region where Melo et al. (2010b) identified this species also in natural environments.

There is no genetic difference between *C. gasar* and *C. brasiliana* (Varela et al., 2007; Melo et al., 2010a; Lazoski et al., 2011; present study), then we agree to use of the first name, which has precedence according to the principle of International Code of Zoological Nomenclature – ICZN.

Lapègue et al. (2002), confirmed through 16S mtDNA and karyological analyzes the Trans-Atlantic distribution of *C. gasar* in West Africa and South America, including in Brazil. According to phylogenetic analysis performed by Melo et al. (2010a) (which used COI - sequencing of the partial cytochrome oxidase and subunit I gene), *C. rhizophorae* and *C. gasar* grouped with *C. virginica*, from the North Atlantic (USA), forming a monophyletic Atlantic group, which was also evidenced by the cladogram generated in the present study, where it is observed the genera *Ostrea* and *Saccostrea* being more basal groups, as also observed by Varela et al. (2007) and for *Ostrea* spp. by Melo et al. (2010a).

In the present study, all the samples obtained in the crops were *C. gasar*. According to Legat et al. (2017), this oyster seems to adapt better in cultivation in relation to *C. rhizophorae,* where it also has a better performance (growth). This was also observed in farms in southern Bahia (Boehs, personal observation), where *C. rhizophorae* has low or no survival, an aspect that needs to be investigated. In Cananéia region (south coast of São Paulo State, southeastern Brazil), *C. gasar* (referred to as *C. brasiliana*) has been successfully cultivated on boards (tables) for decades (Pereira et al., 2001). In this context, Varela et al. (2007) believe that the oysters grown in the north and northeast of Brazil, usually referred to as *C. rhizophorae*, may not usually be of this species. We speculate that perhaps these oysters are *C. gasar*, which was true for the crops investigated in the present study, but this needs to be investigated in other localities.

CONCLUSIONS

The genetic analysis confirmed the existence of two native oysters on the coast of Bahia State: *Crassostrea rhizophorae* ("mangrove oyster") and *C. gasar* ("bottom oyster"), not distinguishable by external shell appearance. The data also indicated that these two species inhabit distinct places within the studied estuaries.

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