

FIRST RECORD OF THE *Saccostrea* OYSTER IN BERTIOGA, SÃO PAULO, BRAZIL

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ABSTRACT

This study presents the first record of an alien species of oyster in Bertioiga, São Paulo State (Southeast Brazilian coast). Alien oysters were found attached to mangrove roots, rock shores, stones and gravel in the riverbed, forming clusters of 10–20 individuals and cohabiting with native oyster species (*Crassostrea mangle*, *C. brasiliiana* and *Ostrea* sp.). Results are presented based on molecular analysis of specimens collected in the Itaguapé River in June 2014. We used partial sequences of 16S and COI genes to assess the taxonomic identity. The Neighbor-joining method was used to analyze phenetic relationships among samples and the genetic diversity was calculated from the Kimura two-parameter (K2P) distances. The sequences in this work clustered with a sequence of "*Saccostrea cucullata*" from Madagascar for both genes (COI and 16S) and presented a genetic distance of 1.7 – 2.2% and 3.5 – 5.3% from other sequences of "*S. cucullata* group" for 16S and COI fragments, respectively. The genetic distances from others *Saccostrea* species (*S. palmula*, *S. glomerata* and *S. mordax*) ranged from 4.7 to 9.1% for 16S and from 13.8 to 19.0% for COI. The genetic distances from other oysters' species sequences (genera *Ostrea* and *Crassostrea*) are over than 14.0% and 25.0% for 16S and COI, respectively. The record is discussed in the context of possible consequences on the environment and probable pathways of introduction. This is the first published record of a *Saccostrea* species in the southwestern Atlantic Ocean.

Key words: *Saccostrea*; bivalve; exotic species; oyster; molecular identification

PRIMEIRO REGISTRO DA OSTRAS *Saccostrea* EM BERTIOGA, SÃO PAULO, BRASIL

RESUMO

Este trabalho apresenta o primeiro registro de uma espécie exótica de ostra em Bertioiga, Estado de São Paulo (sudeste da costa brasileira). As ostras exóticas foram encontradas fixadas em raízes de mangue, costões rochosos, pedras e cascalhos no leito do rio, formando agrupamentos de 10 – 20 indivíduos e coabitando com as espécies de ostras nativas (*Crassostrea mangle*, *C. brasiliiana* e *Ostrea* spp.). Os resultados baseiam-se em análises moleculares de espécimes coletados no rio Itaguapé em junho de 2014. Foram utilizadas sequências parciais dos genes 16S e COI para avaliar a identidade taxonômica. As análises fenéticas foram realizadas pelo método de *Neighbour-joining*. A divergência genética foi calculada através das distâncias do parâmetro-2 de Kimura (K2P). As sequências agruparam-se com uma sequência de *Saccostrea cucullata* de Madagascar para ambos os genes (COI e 16S) e apresentaram uma distância genética de 1,7 – 2,2% e 3,5 – 5,3% de outras sequências do "grupo *S. cucullata*" para fragmentos de 16S e COI, respectivamente. As distâncias genéticas de outras espécies de *Saccostrea* (*S. palmula*, *S. glomerata* e *S. mordax*) variaram de 4,7 a 9,1% para 16S e de 13,8 a 19,0% para COI. As distâncias genéticas de outras espécies de ostras (gêneros *Ostrea* e *Crassostrea*) foram superiores a 14,0% e 25,0% para 16S e COI, respectivamente. O registro é discutido no contexto de possíveis consequências para o ambiente e prováveis vias de introdução. Este é o primeiro registro publicado de uma espécie de *Saccostrea* no sudoeste do Oceano Atlântico.

Palavras-chave: *Saccostrea*; bivalve; espécie exótica; ostra; identificação molecular

Nota Científica: Recebido em 19/06/2017; **Aprovado em** 04/10/2017

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INTRODUCTION

Oysters play an important ecological role in nearshore ecosystems, and have economic significance for local fishing communities (GRABOWSKI *et al.*, 2012). Among the native Brazilian bivalves, representatives of the Ostreidae family include the genera *Ostrea* Linnaeus, 1758, *Crassostrea* Sacco, 1897, and *Dendostrea* Swainson, 1835 (RIOS, 2009). Oysters exhibit high degrees of morphological plasticity and wide geographic distribution. This has led to errors and confusion in their taxonomy because classification and identification of oysters is mainly based on morphological characters (LAM and MORTON, 2006; LIU *et al.*, 2011; LOHAN *et al.*, 2015).

In the last decade, the analysis of DNA sequence data has provided suitable molecular tools for species identification (REECE *et al.*, 2008; SALVI *et al.*, 2014; LOHAN *et al.*, 2015). DNA barcoding based on a standard mitochondrial fragment cytochrome c oxidase subunit I (COI) is a valuable tool in identifying species with high levels of morphological plasticity (HEBERT *et al.*, 2003; SEKINO and YAMASHITA, 2013). Studies involving mitochondrial and nuclear DNA have been conducted to elucidate the taxonomic status of Brazilian *Crassostrea* oysters (IGNACIO *et al.*, 2000; LAPÈGUE *et al.*, 2002; LAZOSKI *et al.*, 2011), and to identify the presence of exotic species, such as Indo-Pacific *Crassostrea* species in northern and southeastern Brazil (VARELA *et al.*, 2007; GALVÃO *et al.*, 2013), and natural *C. gigas* banks on the south coast of Brazil (MELO *et al.*, 2010).

In the present work, we used mitochondrial DNA sequences to assess the taxonomic identity of an alien oyster species found in an estuary located in São Paulo State, on the southeastern Brazilian coast.

METHODS

Oysters were collected from the bottom (2–6 m deep) of the Itaguapé River, Bertioiga, São Paulo State, Brazil (45°57.9'W; 23°46.7'S) (Figure 1), by divers in June 2014. The coastal region where the Itaguapé River flows 12.5 km into the Atlantic Ocean is surrounded by mangrove, Atlantic forest, floodplain forest, and resting vegetation. Biometric data, i.e., height (the dorsal-ventral axis), length (the anterior-posterior axis), and width (the lateral axis), were taken for each oyster with a calliper ± 0.01 mm accuracy (GALTSOFF, 1964). The material was

deposited in the mollusk collection of the Museu de Zoologia da Universidade de São Paulo (MZUSP) receiving the register number MZ 134246.

Samples of the adductor muscle from 15 specimens were placed in 95% ethanol and stocked in a freezer at -20°C . Genomic DNA of each oyster was individually extracted using the Illustra tissue and cells genomic Prep Mini Spin Kit (GE Healthcare, Life Sciences, Buckinghamshire, UK). Two partial mitochondrial fragments, COI and 16S rDNA, were amplified by polymerase chain reaction (PCR) using universal primers designed by FOLMER *et al.* (1994) for the COI gene (LCO1490 5'-GGT CAA CAA ATC ATA AAG ATA TTG G-3' and HCO2198 5' - TAA ACT TCA GGG TGA CCA AAA AAT CA-3') and by PALUMBI (1996) for the 16S gene (16S.AR 5'-CGC CTG TTT ATC AAA AAC AT-3' and 16S.BR 5'-CCG GTC TGA ACT CAG ATC ACG T-3'). PCR was performed in a final volume of 50 μL with $1\times$ reaction buffer, 0.2-mM each dNTP, 1.5-mM MgCl_2 , 0.2- μM of each primer (forward and reverse), 1.0 U PLATINUM[®] Taq DNA Polymerase High Fidelity (Invitrogen[™], Carlsbad, CA, USA), containing 0.5–2 ng DNA. The DNA amplification reaction was conducted in a PTC-200 thermal cycler (MJ Research, Waltham, MA, USA) 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 a final extension at 72°C for 10 min.

The amplification products were purified with the Illustra[™] GFX[™] PCR DNA and Gel Band Purification Kit and eluted in ultrapure water. The purified PCR products were sequenced bi-directionally using the BigDye[®] Terminator Cycle Sequencing kit (Applied Biosystems[™], Carlsbad, CA, USA) according to the manufacturer's protocols. Electrophoresis of the purified samples was performed in ABI PRISM[®] 3100 DNA Sequencer (Applied Biosystems[™], Carlsbad, CA, USA).

Sequences were edited and aligned using CodonCode Aligner v3.5.6 (CodonCode Aligner Corp., Edham, MA, USA). The assembled sequences were deposited in GenBank with the accession numbers KT970455–KT970466. The in silico DNA sequences comparison analysis was performed in MegaBLAST (ZHANG *et al.*, 2000; MORGULIS *et al.*, 2008) to find highly similar sequences. The consensus sequences of each specimen and additional sequences obtained from GenBank were then aligned with ClustalW (THOMPSON *et al.*, 1994)

as implemented in MEGA v6 (TAMURA *et al.*, 2013). The neighbor-joining method was used to analyze phenetic relationships among samples (SAITOU and NEI, 1987). Bootstrap re-sampling analysis was performed to assess support for individual nodes

using 1,000 replicates with 10,000 random additions (NEI and KUMAR, 2000). The genetic diversity was calculated from the Kimura two-parameter (K2P) distances (KIMURA, 1980) using MEGA v.6 software (TAMURA *et al.*, 2013).

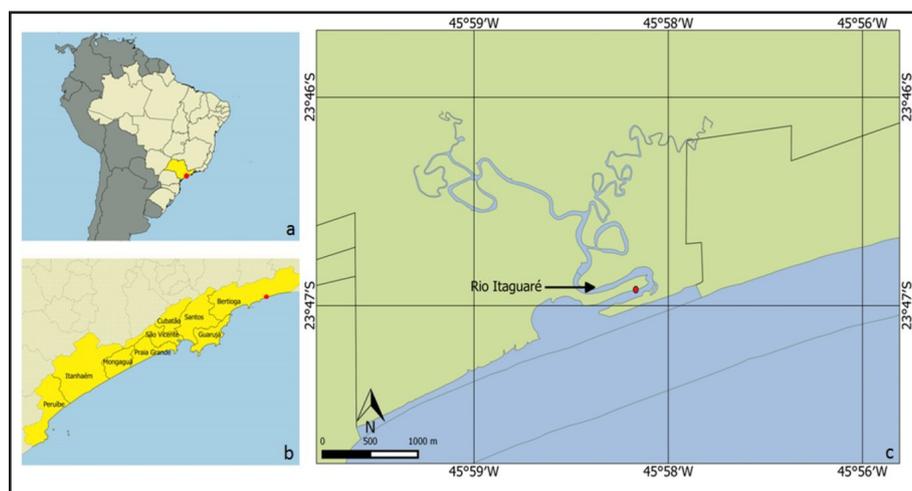


Figure 1 . Map of sampling site location (Elaborated by the authors)

RESULTS

Figure 2 shows the oysters collected for this work. The left valve is a cup shape, with a deep concavity in the umbonal region. Radial ripples can be seen from the umbo to the margins on the outer edge of the left valve. Projections are present along the margin. The right valve is smaller and flat, presenting small denticles along the inner edge, producing corresponding depressions on the left valve. The adductor muscle scar is reniform, and located in the central region. The 15 oysters collected were 56.3 ± 16.8 mm high, 39.9 ± 14.8 mm long, and 18.3 ± 6.9 mm wide (average \pm standard deviation). The sizes ranged from 40 to 98 mm in height, from 22 to 65 mm in length, and from 9 to 30 mm in width.

Based on morphological characteristics described above, we found the alien oysters distributed between 100-2000 m from the Itaguaçu River mouth, attached to mangrove roots, rock shores, stones and gravel in the riverbed, forming clusters of 10-20 individuals and cohabiting with native oyster species, *Crassostrea mangle* Amaral & Simone, 2014,

C. brasiliensis (Lamarck, 1819) and *Ostrea* spp.. Beyond Itaguaçu River, the alien oysters were also found in Guaratuba River ($45^{\circ}53.8'W$, $23^{\circ}45.9'S$) and Bertioga Channel ($46^{\circ}08.6'W$, $23^{\circ}51.4'S$), about 20 km from the collect site.

The neighbor-joining dendrogram, obtained from the K2P distance, shows the genetic distances and clustering of related species (Figures 3 and 4). The bootstrap values indicate the branches' consistency index. The sequences in this work (ScB1, ScB2, ScB3, ScB4, ScB5 and ScB6 - GenBank accession numbers KT970455-KT970466) clustered with a sequence of *Saccostrea cucullata* from Madagascar (KP967577). Nevertheless, they presented a genetic distance of 1.7 - 2.2% and 3.5 - 5.3% from others sequences of "S. cucullata group" for 16S and COI fragments, respectively. The genetic distances from others *Saccostrea* species (*S. palmula*, *S. glomerata* and *S. mordax*) ranged from 4.7 to 9.1 % for 16S and from 13.8 to 19.0% for COI. The genetic distances from other oysters' species sequences (genera *Ostrea* and *Crassostrea*) are over than 14.0% and 25.0% for 16S and COI, respectively.

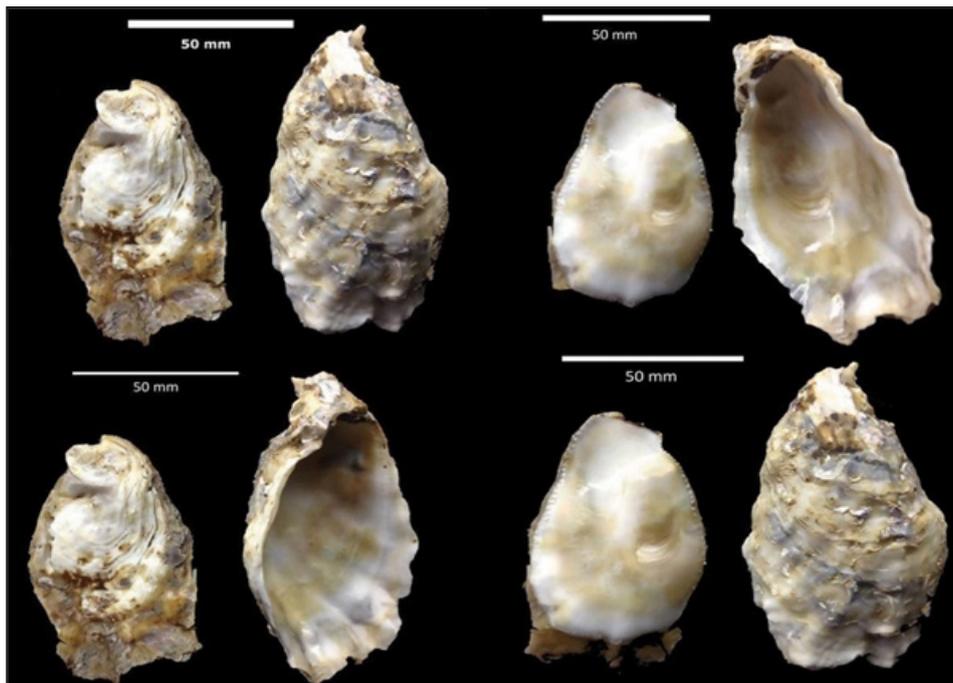


Figure 2. *Saccostrea* specimen (MZSP 134246) collected in Itaguará River, Bertioiga, SP. Actual size: 98 mm

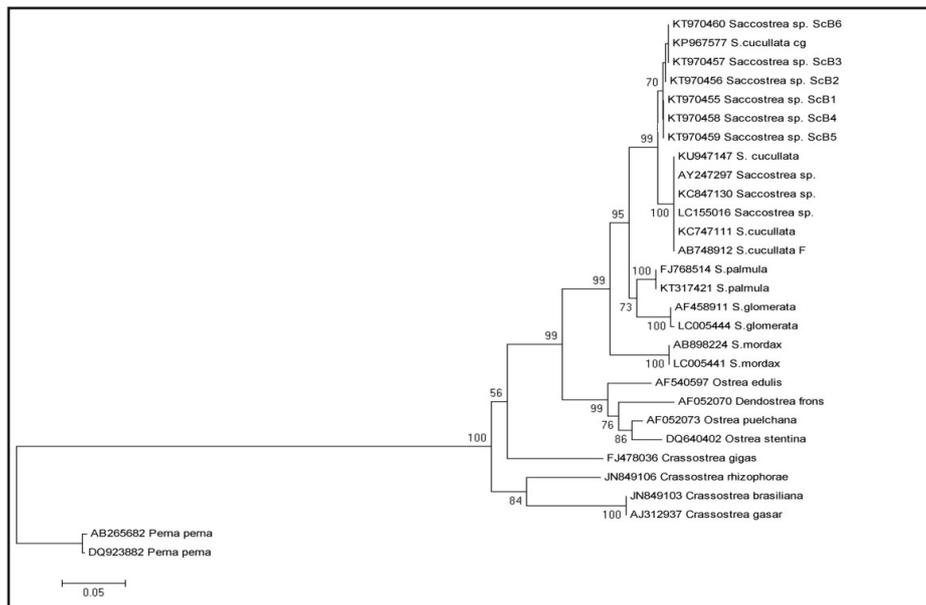


Figure 3. Neighbor-joining dendrogram of the 16S sequences for oysters sampled in Itaguará River, Bertioiga, SP, Brazil. The bootstrap values are indicated on the branches

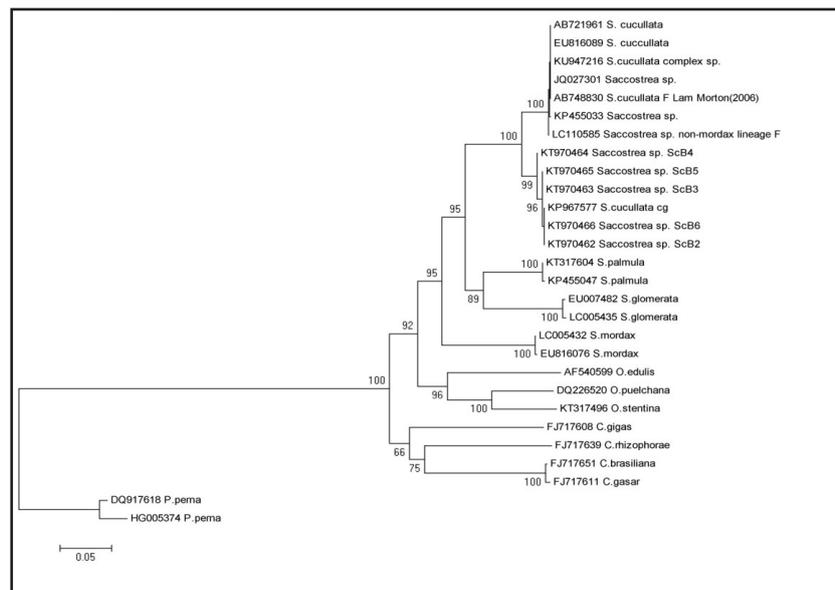


Figure 4 . Neighbor-joining dendrogram of the COI sequences for oysters sampled in Itaguapé River, Bertioga, SP, Brazil. The bootstrap values are indicated on the branches

DISCUSSION

According to these results, the specimens collected on Itaguapé River belong to *Saccostrea* genus, but it is not possible precise which species it belongs. This is the first published record of a *Saccostrea* species in the southwestern Atlantic Ocean.

Species of the genus *Saccostrea* Dollfus & Dautzenberg, 1920, are common oysters that live on rocky shores in the Indo-Pacific Ocean (LAM and MORTON, 2006), but the taxonomy of this group is very controversial. A chaotic taxonomy has resulted from morphological ecotypes being given different names by different authors. According to LAM and MORTON (2006), in the Indo-West Pacific, *Saccostrea cucullata* (Born, 1778) *sensu lato* occupies a wide range of habitats, from mangroves to rocky shores; oyster populations experiencing different degrees of wave exposure and salinity have distinctive ecotypes. This species complex comprises at least seven species, with broadly overlapping geographic distributions (LAM and MORTON, 2006; HAMAGUCHI, 2014). Currently, there are only 10 valid taxonomic species among the dozens of species names for *Saccostrea* (HUBER, 2010). Many molecular analyses and phylogenetics studies have been carried out to elucidate the taxonomy of this group (LIU *et al.*, 2011; HAMAGUCHI *et al.*, 2014; SALVI *et al.*, 2014; LOHAN *et al.*, 2015). *S. cucullata* is believed to have been introduced into a number of locations where it

has become established, including Hawaii (COLES *et al.*, 1999), the Mediterranean Sea (ÇYNDAR *et al.*, 2011), and the Caribbean Sea (LOHAN *et al.*, 2015).

Recent introductions of exotic bivalve species such as *Isognomon bicolor*, *Mytilopsis leucophaeata*, *Crassostrea gigas*, *Myoforceps aristatus* (TEIXEIRA *et al.*, 2010), and *Limnoperna fortunei* (OLIVEIRA *et al.*, 2006) have been reported along the Brazilian coast. Rising concern about the harmful impacts of non-native species has resulted in substantial literature evaluating the risks of bivalve introductions (RUESINK *et al.*, 2005). For instance, the golden mussel, a bioinvasive mollusk that colonizes the freshwater cooling systems of boats (OLIVEIRA *et al.*, 2006) and power generation plants (DARRIGRAN and DRAGO, 2000), has resulted in economic losses in different watersheds in Brazil. Oyster introductions can also impact habitat structure, influencing trophic dynamics and water quality, and by introducing disease-causing organisms resulting in economic, ecological, and social damage (RUESINK *et al.*, 2005; MCKINDSEY *et al.*, 2007).

Global shipping activity has been identified as the main source of species introductions in coastal estuarine and marine habitats. Ballast water and hull fouling are the main vectors associated with this invasion pathway (RUIZ *et al.*, 1997). The area where *Saccostrea* oyster was found is close to the Port of Santos, the largest commercial harbor in Latin America. This area is also one of the most important

petrochemical, chemical, and metallurgical industrial complexes in Brazil. This suggests the presence of *Saccostrea* oyster in the region may be the result of shipping activity in the harbor. LOHAN *et al.* (2015) also suggest that *Saccostrea* sp. have been introduced to the Caribbean Sea by either recreational or commercial vessels due the high connectivity of shipping and boating in the Panama Canal.

Another pathway is the introduction of species outside of their native range for aquaculture. This is one of the most common modes of introduction of exotic bivalves (McKINDSEY *et al.*, 2007). One example is the Pacific oyster, *Crassostrea gigas*, one of the most commonly introduced oyster species for aquaculture in worldwide. This species was introduced in Brazil for aquaculture purpose and today it was found amongst the native species in oyster banks in Brazilian South Coast. However, we do not believe that aquaculture was responsible for the introduction of *Saccostrea* oyster in this particular case.

Previous studies have reported that *Saccostrea* species are well established in the Mediterranean (ÇINAR *et al.*, 2011) and Caribbean Seas (LOHAN *et al.*, 2015). Furthermore, *S. cucullata* and *S. commercialis* are listed in the Delivering Alien Invasive Species Inventories for Europe (DAISIE European Invasive Alien Species Gateway, 2008). These facts raise concerns about this species as a bioinvader, and the potential consequences of this occurrence in the southwest Atlantic warrants further investigation. Therefore, this first occurrence of the *Saccostrea* oyster and its eventual expansion along the Brazilian coast should be monitored in the future.

ACKNOWLEDGEMENTS

To the Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP nº 07/50094-0) for granting funds to support this project, to MSc. Fabrício Gandini, from the Instituto Maramar, for providing the samples and to Dr. Helcio Luis de Almeida Marques for the valuable suggestions.

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