

## MOLECULAR IDENTIFICATION OF NATIVE OYSTERS ON THE COAST OF MARANHÃO, BRAZIL\*

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### ABSTRACT

Oysters are found along the whole coast of Brazil. The phenotypes of the species vary considerably according to the characteristics of the habitat. The present study investigated the existence of different oyster species of the genus *Crassostrea* on the coast of Maranhão, using the Multiplex PCR technique and DNA Barcoding. The results of the Multiplex PCR revealed two distinct bands characteristic of the species *C. gasar* and *C. rhizophorae* in a total of 135 samples analyzed. The sequencing of the COI gene of 98 samples produced a 695 bp fragment and 15 haplotypes for *C. gasar* and 640 bp and eight haplotypes for *C. rhizophorae*. The haplotype tree divided the two species clearly into different clades with 100% bootstrap support. Intraspecific genetic divergence was 0.2% in both species, while interspecific divergence was 23.6%. The similarity between the sequences generated and those available in BoldSystems ranged from 97.01% to 98.37% for *C. rhizophorae* and from 97.55% to 99.84% for both *C. gasar* and *C. brasiliiana*, reinforcing the taxonomic problems in this group, which supports the synonymization of these species. The DNA barcoding permitted the reliable identification of the samples and confirmed the existence of two species of oyster in the study area.

**Key words:** mitochondrial DNA; COI; species identification.

### IDENTIFICAÇÃO MOLECULAR DE OSTRAS NATIVAS NA COSTA DO MARANHÃO, BRASIL

### RESUMO

As ostras são encontradas ao longo de toda a costa do Brasil. Os fenótipos das espécies variam consideravelmente de acordo com as características do habitat. O presente estudo investigou a existência de diferentes espécies de ostra do gênero *Crassostrea* no litoral do Maranhão, utilizando a técnica de PCR Multiplex e DNA *barcoding*. Os resultados da PCR Multiplex revelaram duas bandas distintas, características das espécies *C. gasar* e *C. rhizophorae* num total de 135 amostras analisadas. No sequenciamento do gene COI de 98 amostras obteve-se fragmentos de 695 pb e 15 haplótipos para *C. gasar* e de 640 pb e oito haplótipos para *C. rhizophorae*. A árvore haplotípica agrupou fortemente as duas espécies em cladogramas diferentes com 100% de *bootstrap*. Divergências intraespecíficas foram de 0,2% para ambas, e a interespecífica de 23,6%. A similaridade das sequências deste estudo com sequências presentes no BoldSystems variou de 97,01 a 98,37 para *C. rhizophorae* e de 97,55 a 99,84 tanto para *C. gasar* como para *C. brasiliiana*, reforçando uma problemática na taxonomia do grupo, que apontam para a sinonímia dessas espécies. Portanto, o DNA *barcoding* permitiu identificar as amostras coletadas e confirmou a existência de duas espécies de ostras.

**Palavras-chave:** DNA mitocondrial; COI; identificação de espécies.

### INTRODUCTION

The oysters of the genus *Crassostrea* (SACCO, 1897) are widely distributed and are found attached to substrates such as roots and rocks in the intertidal zone along the whole coast of Brazil (RIOS, 1994). The considerable morphological similarities of the different taxa hamper the reliable identification of these organisms, and contribute to widespread taxonomic uncertainties (RIOS, 1994; IGNACIO *et al.*, 2000; BONDIOLI *et al.*, 2017).

The phenotypic plasticity of the oysters makes the identification of native species extremely difficult, although molecular techniques have been developed to resolve the

taxonomic and zoogeographic problems of the species of the genus *Crassostrea* (IGNACIO *et al.*, 2000; LAPEGUE *et al.*, 2002; PIE *et al.*, 2006; VARELA *et al.*, 2007; REECE *et al.*, 2008; MELO *et al.*, 2010; GALVÃO *et al.*, 2013). The oysters of this genus are the most cultivated, worldwide, given the well-established husbandry technology and their economic importance (WAKAMATSU, 1973; GALVÃO *et al.*, 2017). In Brazil, there have been a number of attempts to develop the commercial farming of native oysters, although most have been unsuccessful, due primarily to the difficulties of obtaining and identifying the seed of species with potential for farming.

Multiplex PCR techniques have been developed specifically for the identification of oysters of the genus *Crassostrea* (LUDWIG *et al.*, 2011; MELO *et al.*, 2013), with the objective of optimizing the taxonomic identification of these organisms, providing an important tool for aquaculture and fishery operations. In addition to this approach, a fragment of approximately 650 base pairs of the 5' extremity of the mitochondrial Cytochrome oxidase subunit I (COI) gene has been adopted as a universal identification system for animal species, including the oysters of the Atlantic Ocean. Each species is normally represented by a unique sequence or a closely similar group of sequences of this gene fragment, which is known as the DNA barcode (HEBERT *et al.*, 2003).

In the area of the present study on the coast of Maranhão, oysters are found throughout the littoral, in natural beds, in both estuarine environments, where they can be found attached to the roots of mangrove trees, and on open beaches, where they are typically attached to rocky outcrops. Previous molecular studies that included samples from this coast (MELO *et al.*, 2010; LAZOSKI *et al.*, 2011; CAVALEIRO *et al.*, 2013) recorded the

presence of only a single oyster species, *Crassostrea gasar*. However, the lack of more detailed studies on the native species and their distribution in the different habitats of the coast of Maranhão represents a drawback for the development of oyster farming in the region and the understanding of the actual status of the local stocks of this important fishery resource.

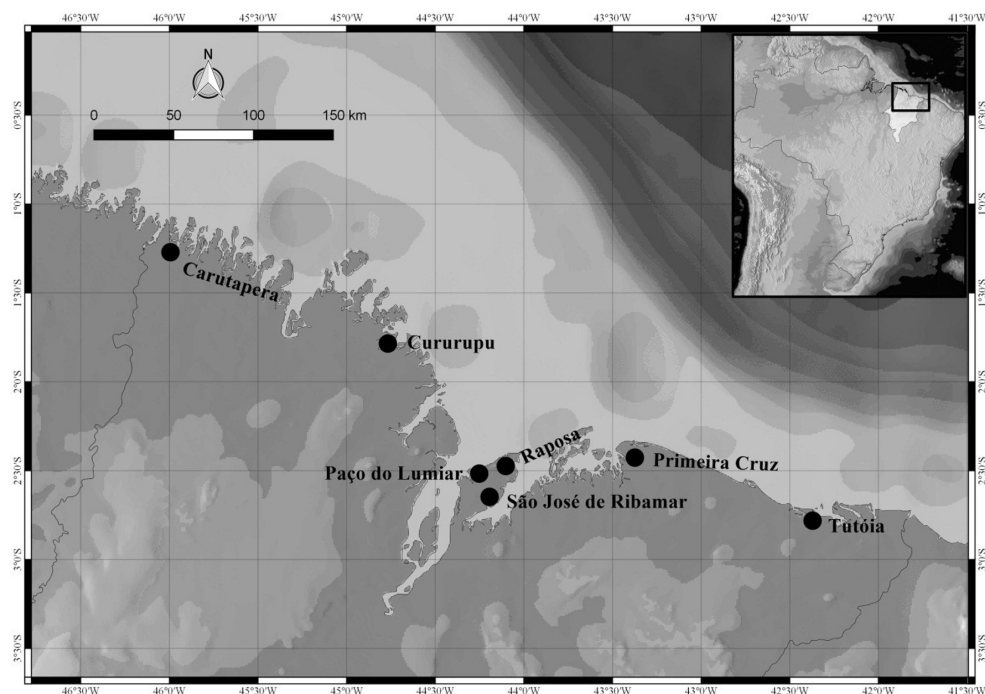
The principal objective of the present study was to identify the native oyster species found on the coast of Maranhão, based on the banding pattern generated by the multiplex PCR and the sequences of the mitochondrial DNA barcoding fragment.

## METHODS

### Samples

The adult specimens were captured in different habitats of the intertidal and subtidal zones, where they were found attached to substrates such as mangrove roots and rocks. The study area encompasses seven sampling points on the coast of Maranhão (Figure 1). Two of the points (Carutapera and Cururupu) are located in the western extreme of Maranhão state, two (Primeira Cruz and Tutóia) are located in the eastern extreme, and three in the center of the state, with samples being collected in 2014 and 2015 (Table 1). The samples were frozen at -20°C and deposited in the tissue and DNA collection of the fauna of Maranhão (COFAUMA) at the Maranhão State University (UEMA) before being transferred to the UEMA Genetics and Pathology Laboratory, where the adductor muscle was removed and preserved in 100% ethanol.

The total DNA was isolated from the tissue of the adductor muscle using MEDRANO *et al.* (1990) saline protocol. The multiplex PCR



**Figure 1.** Map of the sampling points on the coast of Maranhão, Brazil.

**Table 1.** Number of oyster samples collected per collecting point on the coast of Maranhão, Brazil.

MUNICIPALITY	NUMBER OF INDIVÍDUALS	COORDINATES		HABITAT
		S	W	
Raposa	10	2° 26' 25.186"	44° 3' 50.613"	Mangue
São José de Ribamar	20	2° 33' 58.911"	44° 3' 23.519"	Rocha
Paço do Lumiar	25	2° 31' 57.309"	44° 5' 40.923"	Mangue
Primeira Cruz	20	2° 25' 18.3"	43° 25' 01.1"	Mangue
Cururupu	20	1° 47' 17.8"	44° 44' 47.6"	Rocha
Carutapera	20	1° 11' 29.097"	46° 1' 1.074"	Mangue
Tutóia	20	1° 11' 29.097"	46° 1' 1.074"	Mangue
<b>TOTAL</b>	<b>135</b>	-----		

Extraction of the DNA, Multiplex PCR and sequencing of the COI gene.

amplification was based on the protocol and primers described by MELO *et al.* (2013).

The results of the PCR were visualized by horizontal electrophoresis for 80 minutes at 50 V in a 1.5% agarose gel stained with Ethidium bromide. A 1 Kb molecular ladder (1kb Plus DNA Ladder, Life Technologies, Rockville, MD, USA) was used for each migration and the gel was photodocumented for the analysis of the banding pattern and the identification of the *Crassostrea* oysters, as in MELO *et al.* (2013).

The Polymerase Chain Reaction (PCR) was used to isolate and amplify the mitochondrial COI region, using the primers described by FOLMER *et al.* (1994) and MELO *et al.* (2010). The amplification protocol was the same as that used by MELO *et al.* (2010). The PCR products were visualized in 1% agarose gel and purified with ExoSAP-IT following the maker's protocol. The sequencing reaction was based on the SANGER *et al.* (1977) method, using a Big Dye Terminator v3.1 Cycle Sequencing kit (Applied Biosystems). The samples were precipitated in EDTA-Ethanol Sodium Acetate and then sequenced in an ABI 3500 automatic DNA sequencer (Life Technologies) following the manufacturer's recommendations.

## Data analysis

The sequences were edited and aligned using the ClustalW tool (THOMPSON *et al.*, 1994) in the BIOEDIT program, version 7.0.5.2, (HALL, 1999). The data matrix, mean genetic distances, and phylogenetic analyses were run in MEGA 6.0 (TAMURA *et al.*, 2013), using the neighboring-joining approach, with the Kimura 2-parameter model (SAITOU and NEI, 1987). A bootstrap analysis (FELSENSTEIN, 1985) was used to verify the significance of the groupings. The DnaSP program, version 5 (LIBRADO and ROZAS, 2009) was used to analyze haplotype and nucleotide diversity. Molecular identification using the COI gene was based on comparisons of the sequences obtained in the present study with those available on the BOLDSYSTEMS (Barcode of Life Data Systems) platform (HEBERT *et al.*, 2003). A sequence of the COI gene of *Crassostrea* sp. Canela (HM003525) obtained from

the region of Bragança, in Pará, northern Brazil, was incorporated into the database as the outgroup.

The sequences of *C. gasar* (HM003499, HM003507, HM003515, HM003516, HM003517, HM003518 and HM3519) recorded by MELO *et al.* (2010) and those of *C. brasiliiana* (FJ7640, FJ717641, FJ717642, FJ717643, FJ717644, FJ717645, FJ717646, FJ717647, FJ717648, FJ717649, FJ717650 and FJ717651) obtained by LAZOSKI *et al.* (2011) were included in the database. We also included COI sequences of the African species *C. gasar* (FJ717611), obtained from GenBank.

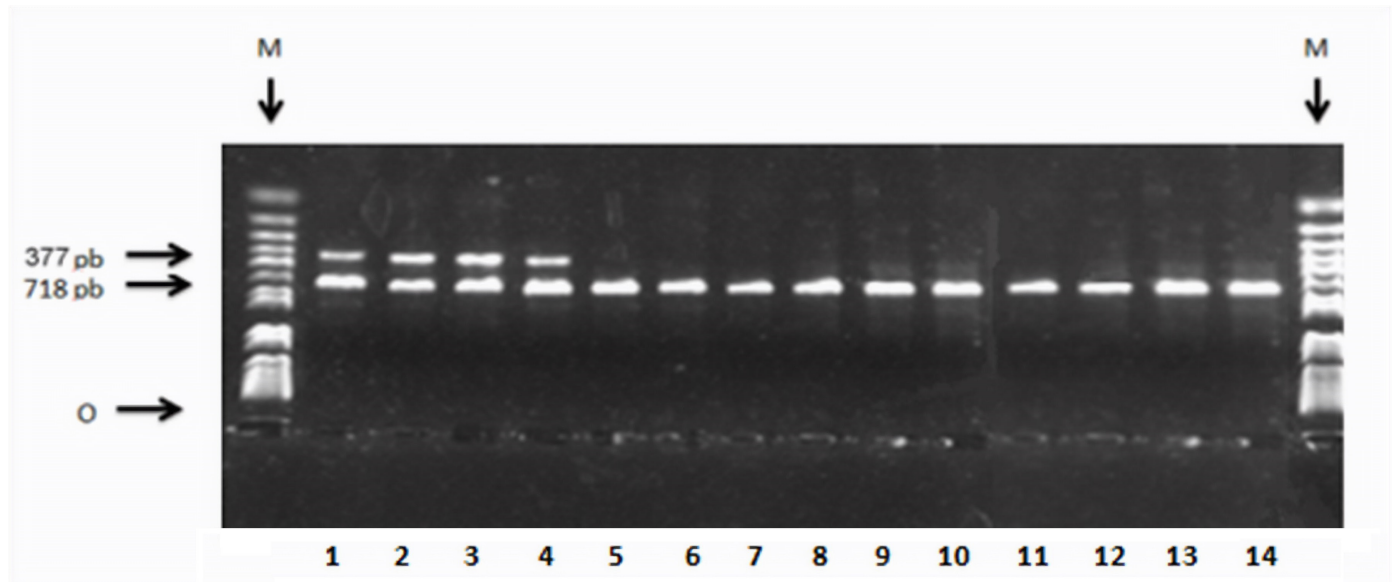
## RESULTS

### Multiplex PCR

A total of 135 individuals were analyzed by multiplex PCR. The bands revealed by the amplification in agarose gel corresponded to those of the species *C. gasar* and *C. rhizophorae* (Figure 2). The bands obtained from the samples from the municipalities of Carutapera, Cururupu, Primeira Cruz and Tutóia were all of 718 bp, corresponding to the ITS 1 region, indicating that *C. gasar* is present at these localities. In the samples from the municipalities of São José de Ribamar, Paço do Lumiar and Raposa, however, two banding patterns were obtained. One was the single band characteristic of *C. gasar*, while the other was a double band, with a 377 bp sequence for the COI region, and 718 bp corresponding to the ITS 1, confirming the presence of *C. rhizophorae* at all three sampling points.

### Sequencing of the COI gene

A total of 98 sequences were obtained from the samples of *Crassostrea* oysters, of which, 78 corresponded to the species *C. gasar*, with a fragment of 695 bp, and 20 were consistent with *C. rhizophorae*, with a fragment of 640 bp. Eight haplotypes were recovered from the *C. rhizophorae* samples, with haplotype diversity (*h*) of 0.795 and nucleotide diversity ( $\pi$ ) of 0.002. In *C. gasar*, a total



**Figure 2.** Image of a 1% agarose gel stained with ethidium bromide showing the amplifications obtained by the Multiplex PCR (samples from São José de Ribamar, Paço do Lumiar and Raposa: 1-4, corresponding to the species *C. rhizophorae*; samples from Carutapera, Cururupu, Primeira Cruz and Tutóia: 5-14, corresponding to the species *C. gasar*; M = DNA Marker).

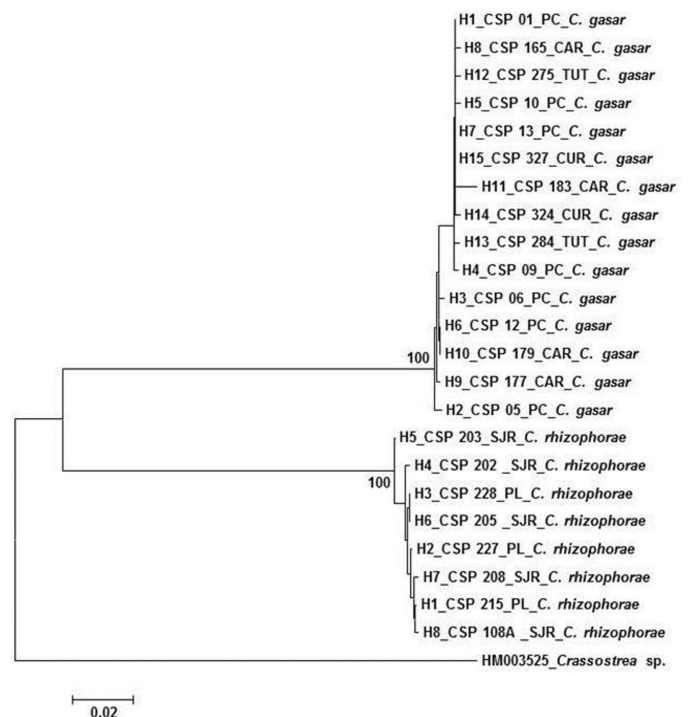
of 15 haplotypes were identified, with haplotype diversity ( $h$ ) of 0.428 and nucleotide diversity ( $\pi$ ) of 0.002.

The haplotype tree based on the Neighbor Joining (NJ) approach and the Kimura 2-parameter (K2P) model presented two strongly-supported clades. All the *C. rhizophorae* haplotypes were grouped in a basal clade (100% bootstrap support), while the *C. gasar* haplotypes constituted a second, clade with equally strong support (Figure 3).

The clear separation of *C. rhizophorae* and *C. gasar* in distinct groups confirms the occurrence of two *Crassostrea* species in the study area on the coast of Maranhão, corroborated by the high genetic divergence values (23.9-25.9%) recorded between the haplotypes of the two taxa. A mean intraspecific genetic divergence of 0.20% was recorded for both the oyster species, whereas the divergence recorded between *C. gasar* and *C. rhizophorae* was 23.6% (Table 2). The existence of distinct *C. rhizophorae* and *C. gasar* populations is thus further supported by the high genetic divergence value recorded between these two taxa.

### Molecular identification

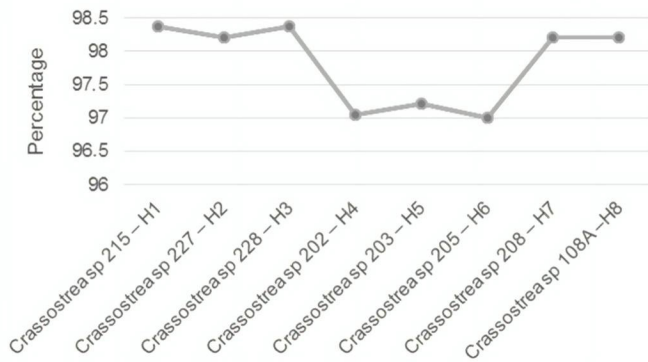
The COI sequences were submitted to the BOLDSYSTEMS platform for the identification of the samples through comparisons with those deposited in this system. Sequences identified as *C. rhizophorae* were 97.01% to 98.37% similar to the sequences of this species available in BOLDSYSTEMS (Figure 4). In the case of the samples identified as *C. gasar*, similar percentages of similarity, varying from 97.55% to 99.84%, were found in relation to the BOLDSYSTEMS sequences of both *C. gasar* and *C. brasiliiana*, raising a taxonomic conundrum (Figure 5). These



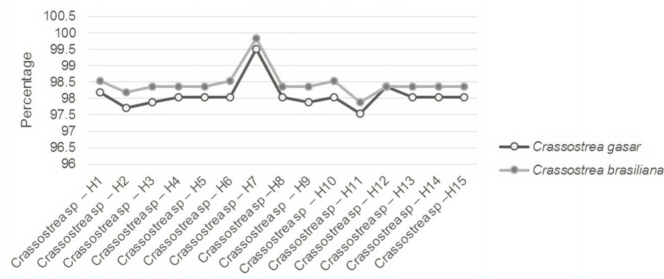
**Figure 3.** Haplotype tree produced by the neighbor joining (NJ) method using the K2P model based on the sequences of the COI gene in oysters of the genus *Crassostrea* in this study and GenBank sequences. The numbers at the nodes represent the bootstrap values (1000 replicates). CAR = Carutapera; CUR = Cururupu; PC = Primeira Cruz and TUT = Tutóia; SJR = São José de Ribamar; and PL = Paço do Lumiar; CSP = *Crassostrea* sp.

**Table 2.** Intra- and interspecific genetic divergence (K2P) in the oysters of the genus *Crassostrea* from the coast of Maranhão, Brazil.

Category	Species	N	(%) K2P Divergence
			Mean
Intra - specific	<i>C. rhizophorae</i>	20	0.20
	<i>C. gasar</i>	78	0.20
Inter - specific	-	98	23.6



**Figure 4.** Percentage genetic similarity of the *C. rhizophorae* haplotypes obtained by molecular identification in the BOLDSYSTEMS platform.



**Figure 5.** Percentage genetic similarity of the *C. gasar* haplotypes obtained by molecular identification in the BOLDSYSTEMS platform.

results are corroborated by the haplotype tree generated using the GenBank sequences of *C. brasiliensis*, which groups with *C. gasar* with 100% bootstrap support, indicating the formation of a single clade (Figure 3).

## DISCUSSION

The results of the Multiplex PCR confirmed the occurrence of *C. rhizophorae* in the study area, on the Maranhão coast, where it had not been recorded previously. Up to now, only *C. gasar* had been recorded in Maranhão (MELO *et al.*, 2010; LAZOSKI *et al.*, 2011). The two species were found coexisting

sympatrically on the same stretch of coast in the municipalities of São José de Ribamar (on rocky substrates) and Paço do Lumiar (in mangrove habitats). Sympatry between two or more native species of oyster has been recorded in some other Brazilian estuaries (PIE *et al.*, 2006; GARDUNHO *et al.*, 2012).

The presence of more than one native oyster species on the coast of Maranhão reinforces the need for more detailed studies in reproductive biology and the evaluation of the stocks of both species. As two species are now known to occur in the region, previous evaluations of the status of this fishery resource would be incomplete, given that they considered the presence of only a single species in the area.

In the study region, oysters are typically harvested in a rudimentary way by the traditional local communities, with no concern for the management of the resource or its sustainability. Scientific studies that provide reliable data on the status of stocks provide the potential for the implementation of effective management measures by these local communities. Sustainable techniques, such as oyster farming (CAVALLI and FERREIRA, 2010), represent an economically viable alternative source of subsistence for the populations that depend on this natural resource. However, the correct identification of the native species found in a given area is a fundamental first step in the development of effective oyster farming operations.

Even though all the native species of *Crassostrea* oysters can be farmed commercially, *C. gasar* has been shown to perform best, in terms of captive growth rates, indicating that the cultivation of this species may be the most lucrative in most cases (CHRISTO and ABSHER, 2006). In this case, the mapping of the distribution of the two native species found in the study area will be of fundamental importance for the effective recruitment of oyster seed. There are a number of points on the coast of Maranhão – Carutapera, Primeira Cruz, Cururupu and Tutoia – that have considerable potential for the recruitment of oyster seed, given that only *C. gasar* was found in these areas. However, new studies need to focus on the genetic identification of specimens and the recruitment of oysters to confirm this conclusion.

The findings of the present study have extremely important implications for the development of oyster farming and harvesting operations on the Maranhão coast, given the general lack of data from this region. While this region has an enormous potential for oyster farming, there is no captive production whatsoever, which reinforces the need for the optimization of the points for the recruitment of native oyster seed.

While the Multiplex PCR was regulated for the identification of up to four oyster species, one (*C. gigas*) was not expected for the study area. This is because *C. gigas*, an exotic species from the Indo-Pacific region, would not be expected to tolerate the high temperatures typical of the area of the present study (MELO *et al.*, 2010). The Multiplex PCR proved effective for the identification of the *Crassostrea* species. The efficiency of this approach indicates that it may be a practical alternative for the monitoring of the distribution of oyster species, as well as being an accessible tool for the oyster farmer.

In the analysis of the sequences of the COI gene, the clear separation of *C. rhizophorae* and *C. gasar* into two distinct groups confirms the occurrence of two *Crassostrea* species on the coast of Maranhão, which is reinforced by the high levels of divergence (23.9% to 25.9%) between the haplotypes of the two taxa. The structure of the haplotype tree obtained in the present study is similar to those obtained by MELO *et al.* (2010) and LAZOSKI *et al.* (2011) using the COI marker, as well as those reported by LAPÊGUE *et al.* (2002) and VARELA *et al.* (2007) for the 16S rRNA gene.

The indices of interspecific divergence recorded in the present study were similar to those obtained by MELO *et al.* (2010), who recorded a value of 26.1% between the same two oyster species. LAZOSKI *et al.* (2011) also obtained values of 16% to 27% for comparisons between *Crassostrea* species, using the same marker (COI). Molecular approaches to the identification of species depend on their capacity to distinguish intraspecific variation from interspecific differences (CYWINSKA *et al.*, 2006). The values found in the present study in *C. gasar* and *C. rhizophorae* further validate the DNA Barcode for the identification and discrimination of oyster species, given that the effectiveness of this approach depends on the existence of a much greater similarity in the DNA sequences within rather than between species (CARVALHO *et al.*, 2008).

The results of the present study, together with those of previous analyses of the same fragment of the COI gene in South American populations of *Crassostrea* oysters (MELO *et al.*, 2010; LAZOSKI *et al.*, 2011), provide important insights into the identification of these species, and the conservation and management of this important fishery resource. The molecular threshold established by the Consortium for the Barcode of Life (CBOL) for the delimitation of a species is 2-3% of genetic divergence. In practice, this means that, if the DNA sequence of a sample differs by less than 3% (or is more than 97% similar in BOLDSYSTEMS) from that of a known species, it should belong to that species (SOLÉ-CAVA and WÖRHEIDE, 2007; SOLÉ-CAVA, 2008). While this threshold is intended for vertebrates, MELO *et al.* (2010) recorded values of approximately 2% between *Crassostrea* species (*C. gigas* and *C. angulata*).

As AMARAL and SIMONE (2014) considered the voucher material of Varela *et al.* (2007) to represent *C. brasiliiana*, the sequences obtained in the present study were compared with those available in Genbank for African *C. gasar* and *C. brasiliiana*. The haplotype tree grouped *C. brasiliiana* and *C. gasar*, indicating that they are in fact a single species. VARELA *et al.* (2007) pointed out that the sequences obtained from *C. brasiliiana* by PIE *et al.* (2006) and from *C. gasar* by LAPÊGUE *et al.* (2002) did in fact belong to the same species. However, AMARAL and SIMONE (2014) consider *C. brasiliiana* and *C. gasar* to be different taxa, the former being a native species and the latter, does not occur in Brazil. While the present study does not include morphological analyses, as in SIMONE and AMARAL (2014), the results of the present study are consistent with those of other recent studies, which confirmed this taxonomic arrangement (MELO *et al.*, 2010; LAZOSKI *et al.*, 2011; CAVALEIRO *et al.*, 2013), indicating that *C. gasar* and *C. brasiliiana* are a single species.

## CONCLUSION

The Multiplex PCR and barcoding approaches adopted in the present study were effective for the identification of the oyster species. The oysters *C. gasar* and *C. rhizophorae* were identified as being native to the coast of Maranhão, where *C. gasar* is amply distributed, occurring at all the sampling points, and *C. rhizophorae* is restricted to the central zone of the littoral of Maranhão.

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