PCR-RFLP FOR IDENTIFICATION OF THE PEARL OYSTER *Pinctada imbricata* FROM BRAZIL AND VENEZUELA

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ABSTRACT

Oysters of the genus *Pinctada* are of great economic importance due to their extensive use in human feeding and pearl cultivation. It includes four species: *Pinctada radiata* (Europe), *P. imbricata* (Western Atlantic) and the *P. fucata-martensi* complex (Pacific), the latter being a species complex of difficult morphological differentiation. Although this species complex has several molecular studies corroborating each species as valid, there are still doubts about the validity of *P. imbricata* in the South of Western Atlantic (ie Brazilian Coast). Here we carried out a RFLP study with populations from Ceará, Rio de Janeiro, São Paulo and Venezuela coast. We analyzed mitochondrial (16S) and nuclear genes (partial IGS). This study confirms the Brazilian and Venezuelan stocks as genetically close to the *P. imbricata* stocks from Caribbean than the *P. martensi-fucata* complex. This result is important for pearl-oyster farmers, demonstrating that the Brazilian and Venezuelan stocks are not alien species or hybrids of Indo-Pacific species.

Key words: oyster, *Pinctada imbricata,* South America, Pearl oyster

PCR-RFLP PARA IDENTIFICAÇÃO DE OSTRAS PERLÍFERAS (*Pinctada imbricate*) DO BRASIL E VENEZUELA.

RESUMO

As ostras do género *Pinctada* são de grande importância económica devido ao seu uso extensivo na alimentação humana e cultivo de pérolas. Inclui quatro espécies: *Pinctada radiata* (Europa), *P. imbricata* (Atlântico Ocidental) e *P. fucatamartensi* (Pacífico), sendo esta última uma espécie complexa de difícil diferenciação morfológica. Embora este complexo de espécies tenha vários estudos moleculares corroborando cada espécie como válida, ainda há dúvidas sobre a validade de *P. imbricata* no Sul do Atlântico Ocidental. Neste trabalho realializou-se um estudo de RFLP com populações do Ceará, Rio de Janeiro, São Paulo e costa da Venezuela. Foram analisados genes mitocondriais (16S) e nucleares (IGS parcial). Este estudo confirma que os espécimes brasileiros e venezuelanos são geneticamente mais próximos às populações de *P. imbricata* do Caribe do que o complexo *P. martensi-fucata*. Esse resultado é importante para os produtores de ostras perlíferas, demonstrando que os estoques brasileiro e venezuelano não são espécies exóticas ou híbridas de espécies indo-pacíficas.

Palavras-chave: Ostras, *Pinctada imbricata,* América do Sul, ostra perlífera

Nota Científica: Recebido em 25/01/2017; **Aprovado em** 31/07/2017

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INTRODUCTION

The Atlantic pearl oyster, *Pinctada imbricata* (RODING, 1798), is a relative common filter-feeding epibenthic bivalve and one of the most biologically and economically important species belonging to the family Pteriidae, given its extensive use in human feeding and pearl cultivation (URBAN, 2000; VILLALOBOS and ELGUEZABAL, 2001; MARQUES and BARBIERI, 2015). Though some authors state that its southernmost occurrence is Venezuela (SHIRAI, 1994; O' CONNOR and LAWLER, 2004; WADA and TEMKIN, 2008; see also the World Register of Marine Species database, BOUCHET and ROCROIT, 2013). *P. imbricata* is alternatively reported to range along the Western Atlantic: from North Carolina (USA) to the southern Caribbean and southern Brazil (MARCANO *et al*., 2005; RIOS, 2009), with occasional records from the Uruguayan coast (MARQUES and BREVES, 2015).

Questions concerning the identity of *P. imbricata* and the Pacific and Mediterranean congeners (*P. fucata-martensi* and *P. radiata*, respectively) are plentiful. Most of the confusion stems from intra (and inter) specific polymorphism, geographical isolation of some populations, transportation, hybridization, and erratic taxonomical practice (WADA and TEMKIN, 2008). SOUTHGATE (2007) defined the "Akoya pearl oysters" as a species complex that encloses *P. imbricata* from America and *P. fucata* and *P martensii* from the Japanese and Pacific coasts. TEMKIN (2010) expanded that concept by allocating *P. radiata* from Europe in a species complex termed *P. fucata/martensii/radiata/imbricata* group.

SOUTHGATE (2007) pointed out a possible hybrids from interbreeding of *P. imbricata* imported stocks and the local *P. martensi* in Japan Coast. MASAOKA and KOBAYASHI (2005) have shown by molecular identification that the *P. imbricata* from Caribbean and *P. fucata* from Pacific populations are actually distinct species (although *P. fucata* and *P. martensi* did not present molecular differences). Subsequent molecular studies (YU and CHU, 2006; TEMKIN, 2010; CUNHA *et al*., 2011) corroborated the Masaoka and Kobayashi study, but without addressing the differentiation among *P. radiata* and *P. imbricata.* Although the aforementioned studies are comprehensive in molecular markers, none included South American samples of *P. imbricata*.

Mitochondrial and nuclear markers have been used to show population variability in many species, as well as an important tool for distinguishing marine invertebrate species (ALFAYA *et al.,* 2013; GUZMÁN *et al.,* 2011; YEDNOCK *et al.,* 2014; DI BIASI *et al.,* 2016), including many pteriids (ARNAUD *et al.,* 2000; ARNAUD-HAOND *et al.,* 2005; CUNHA *et al*., 2011; GWAK and NAKAYAMA *et al*., 2011). The PCR-RFLP methods, with rapid visualization on gel, is a quick and efficient tool for verification of specific identities in widely spread marine organisms. In the present paper, we present the results of the restriction sites *Mse I* and *Alu I* (based on MASAOKA and KOBAYASHI's 2005 protocol, for 16S and IGS genes) used to identify specimens of *Pinctada* from Brazil and Venezuela.

METHODS

Samples from three Brazilian sites, Jijoca (2°48'0"S, 40°30'0"W; Ceará State), Ilha Grande (23°08'36,83"S 44°16'09"W; Rio de Janeiro State) and Caraguatatuba (23°36'36,37''S 45°18'51" São Paulo State) and seven sites from Cariaco Golf (10°30'0"N 64°0'0"W), Venezuela, were obtained during in 2012-2013. Abductor muscles were removed from specimens and stored in 70% ethanol. Tissues were digested with Proteinase K (10mg mL^{-1}) at 65°C overnight. DNA extraction was conducted according to ALJABANI and MARTINEZ (1997). Polymerase chain reactions (PCR) used the forward and reverse primers to amplify the 720-bp nuclear IGS region, as described by MASAOKA and KOBAYASHI (2005). Mitochondrial 530-bp 16S gene fragments were amplified using the universal primers 16SAR and 16SBR. PCR products (10µl) were screened with the restriction enzimes *Mse I* and *Alu I,* and directly digested with 0.5 to 2 U of each enzyme, respectively, adding to a final volume of 15µL. Reaction products were separated by electrophoresis on a 3.0% agarose gel in TAE buffer at 100 Volts, and stained with Gel RedTM (Biotium). Restriction fragment sizes were determined by comparison with a 100-bp DNA ladder (Promega). Presence or absence of restriction sites were inferred from fragment patterns.

RESULTS

A total of 111 specimens of *Pinctada imbricata* were sampled*:* 44 from Caraguatatuba (state of São Paulo), 37 from Jijoca (state of Ceará), 24 from Rio

de Janeiro city, and 7 from the Venezuelan Gulf. All individuals were submitted to RFLP analyses. Results are shown on Table 1. All of the specimens have the same pattern of *P. imbricata* haplotypes from the Caribbean – two bands for *Alu I* digestion of 16S gene and four bands for *Mse I* (IGS gene). The three band pattern for *Mse I* digestion of IGS is consistent with the "haplotype B" of Caribbean specimens, as referred to by MASAOKA and KOBAYASHI (2005). *Alu I* did not produce digestion for the IGS gene on *P. imbricata* specimens from Brazil and Venezuela, differently from specimens from the Caribbean, which exhibited a double-banded pattern. *Mse I* digestion of 16S resulted in a large amount of fragments, which were undistinguishable on gel. The *P. martensi-fucata* complex also has two fragments for *Alu I* digestion of the 16S gene, but the larger fragment is less than 400-bp.

Table 1. Pattern of fragments in PCR-RFLP analysis using *Alu I* and *Mse* on IGS and 16S genes. Bolded numbers represent the number of fragments on gel visualization; Size of each fragment in parenthesis.

	SEQUENCES/RFLP			
	IGS		**16S**	
Species	*Alu I*	*Mse I*	*Alu I*	*Mse I*
Pinctada martensi-fucata (GenBank Sequences)	**2** (459;266)	A:**1**(725)	**2** (341;183)	multiple
Pinctada imbricata Caribbean (GenBank sequences)	**2** (455; 265)	B: **3** (326; 232;121) C: **4**(232;174;171;144)	**2** (468;58)	multiple
Pinctada imbricata Brazil and Venezuela	**1**(720)	**4**(~326; ~230; ~120;)	**2** (~470; ~60)	multiple

DISCUSSION

In spite of some authors already described low levels of genetic variability (ARNAULD *et al*., 2000), the lack of polymorphism does not agree with the observations made on other bivalve species with the same markers, whose demographic data strongly suggests to be the result of recent founder event (HUVET *et al*., 2000; ARNAUD-HAOND *et al*., 2005) or bottlenecks (ARNAUD *et al*., 2000) and cannot be attributed to mutation rate of genes analyzed.

Variation within and between populations and stock discrimination within exploited species are important issues in fisheries management and for conservation programmers (LIU and CORDES, 2004). Many non-genetic methods of stock discrimination are available and achieve varying degrees of success in distinguishing breeding stocks. With the advent of genetic methods, stock identification based solely upon morphological and meristic differences has become rare. Instead, these data are used in conjunction with genetic data providing a global view that permit research and farmers working together to achieved better levels of development of its culture with the lowest possible environmental impact.

MASAOKA and KOBAYASHI (2005) pointed the PCR-RFLP method with the present markers as ideal to identify *Pinctada* species. This holds true even for almost morphologically indistinguishable *P. fucata/ martensii/radiata/imbricata* complex (*sensu* Temkin 2010) – particularly the restriction profiles of *Mse I* on IGS and *Alu I* on 16S, as the differences on fragment lengths seem to be species-specific. MASAOKA and KOBAYASHI (2005) did not comment the restriction profile of *Alu I* on IGS. As we analyzed sequences from GenBank, we have found that there is one *Alu I* site (GenBank accession numbers AB214295.1; AB214294 and AB214296.1), whereas the specimens from Brazil and Venezuela resulted in a single fragment (720).

In species having a well-established economic importance, such as the Akoya oysters on the Japanese coast (MARTINEZ-FERNANDEZ and SOUTHGATE, 2007), a quick and low cost molecular technique is useful to investigate the existence of undesirable alien species in farming. Undesired alien species can be brought in by ballast water or even accidentally introduced. The results presented here confirm the MASAOKA and KOBAYASHI (2005) protocol as a useful tool for *Pinctada* species

identification. Likewise, the results indicate that the *P. imbricata* stocks in Venezuela and Brazil stocks are more related to the Caribbean stocks than the *P. martensi-fucata*. Furthermore, the results show that the farming-specimens and seed on both the Brazilian and Venezuelan coasts (as the Caraguatatuba or Venezuela population) are not considered alien species from the Indo-Pacific species. So, this study provides to the pearl-oysters farmers the opportunity to cultivate and exchange specimens from other areas of the Western Atlantic, with low risk of accidental introduction of invasive species.

ACKNOWLEDGEMENTS

This study was supported by a FAPESP grant (Process Number 2012/50184-8) and was possible by an IBAMA – SISBIO permission (Number 37666-1). We are indebt.

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