THE CONTRIBUTION OF GENETICS IN THE STUDY OF THE SEA-BOB SHRIMP POPULATIONS FROM THE BRAZILIAN COAST*

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ABSTRACT

Genetic studies can provide several important informations for sustainable fisheries management, mainly for the identification of different genetic stocks. In Brazil, genetic studies of the sea-bob shrimp, *Xiphopenaeus kroyeri,* produced important new information on the systematics of the genus, demonstrating the presence of anonymous cryptic species and outlining their geographical distribution and the levels of genetic diversity and population structuring of Brazilian species. Allozymes and sequence polymorphisms of the mitochondrial COI gene have also been used to beget diagnosis systems for the correct species identification. Recently, geometric morphometrics emerged as a powerful methodology for identifying operational taxonomic units, confirming genetic data with great similarity of results, and representing a promising tool to assist the identification of fishery stocks. Geometric morphometrics analyses were used to characterize *Xiphopenaeus* samples from Brazil, showing significant differences among populations, confirming previous patterns revealed by molecular data. This study gathers the information produced so far about the molecular systematics, patterns of geographic distribution of the cryptic *Xiphopenaeus* species, genetic and morphometric characterizations of variability and delineation of population boundaries. It intends to determine distribution and structuring consensus patterns of the genetic variability, aiming to support management and conservation actions as well as to identify gaps of knowledge and direct future efforts in order to address them.

Keywords: *Xiphopenaeus kroyeri*; *Xiphopenaeus riveti*; cryptic species; molecular systematics; geometric morphometrics; fisheries genetics

A CONTRIBUIÇÃO DA GENÉTICA NO ESTUDO DAS POPULAÇÕES DE CAMARÃO SETE-BARBAS DO LITORAL BRASILEIRO

RESUMO

Estudos genéticos podem fornecer diversas informações importantes para o manejo sustentável da pesca, tendo um relevante papel na identificação de estoques geneticamente distintos. No Brasil, estudos genéticos do camarão sete-barbas, *Xiphopenaeus kroyeri*, têm produzido novas e importantes informações sobre a sistemática do gênero, revelando espécies crípticas anônimas e delineando suas distribuições geográficas e os níveis de diversidade genética e estruturação populacional das espécies brasileiras. Alozimas e polimorfismos de sequência do gene mitocondrial COI também têm sido usados para gerar sistemas de diagnóstico para a identificação de espécies. Recentemente, a morfometria geométrica surgiu como uma metodologia poderosa para a identificação de unidades taxonômicas operacionais, confirmando os dados genéticos com grande similaridade de resultados, e representando uma ferramenta promissora para auxiliar na identificação dos estoques pesqueiros. Análises de morfometria geométrica foram utilizadas para caracterizar amostras de *Xiphopenaeus* do Brasil, mostrando diferenças significativas entre as populações, confirmando padrões anteriores revelados por dados moleculares. Este trabalho reúne as informações já produzidas sobre a sistemática molecular, padrões de distribuição geográfica das espécies crípticas de *Xiphopenaeus*, caracterização genética e morfométrica da variabilidade e delimitação de populações, a fim de determinar padrões consensuais de distribuição e estruturação genética, com o objetivo de subsidiar ações de manejo e conservação, e também para identificar lacunas de conhecimento e direcionar esforços futuros para resolvê-las.

Palavras chave: *Xiphopenaeus kroyeri*; *Xiphopenaeus riveti*; espécies crípticas; sistemática molecular; morfometria geométrica; genética pesqueira

Artigo de Revisão: Recebido em 06/09/2013 – Aprovado em 28/01/2013

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* *Finnancial support: CNPq*

INTRODUCTION

The main goal of genetic studies in fisheries is the conservation of genetic variability of populations, which is an intrinsic component of biodiversity (ANTONOVICS, 1990). The depletion of the population diversity can lead to decreased sustainability, predisposing populations to reductions in abundance and the collapse of fish populations (MUSTAFA, 1999), even leading to local extinctions of over-exploited stocks (OVENDEN, 1990).

Genetic studies can provide important information for sustainable fisheries management, as the presence of anonymous morphologically similar species (cryptic species), migration rates and effective population sizes. One of the main roles of genetics has been the identification of genetic breeding units (THORPE *et al.*, 2000) i.e., the delimitation of different genetic stocks (*sensu* CARVALHO and HAUSER, 1994). Molecular markers allow managers to identify whether fishery samples come from the same or different populations and verify the relative contribution of different genetic stocks to a mixed fishery stock. This is done based on the analysis of individual genotypes of specimens derived from stocks, for several loci, which may correspond to expressed genes (e.g. isozymes) or not expressed regions of the genome (e.g. microsatellites, mitochondrial control region). The raw genotypic data of the various loci are used for estimation of genotypic and allelic frequencies of the studied populations. Sample frequencies are compared using statistic tests, and significant differences between populations are interpreted as indicative of population structuring (KAPUSCINSKI and MILLER, 2007).

The population differentiation is traditionally measured using the Wright's fixation index (F_{ST}). The F_{ST} measures the proportion of genetic variation that is due to differences between populations. The index ranges from 0-1, and higher values indicate greater differentiation. Several statistical frameworks for the study of small samples derived from a restricted number of populations and F_{ST} analogues have been described, which are more suitable for the analysis of molecular markers with higher levels of polymorphisms (for a review see MEIRMANS and HEDRICK, 2011).

In Brazil, the first efforts to genetically characterize penaeid shrimp species by means of molecular markers began in the 90's (WEBER *et al.*, 1993; MAGGIONI, 1996), employing protein polymorphysms, and the first studies based on DNA polymorphisms date from 2000 (GUSMÃO *et al.,* 2000; MAGGIONI *et al.*, 2001); but none of those works focused on *Xiphopenaeus kroyeri* populations.

The geminal studies addressing the genetic characterization of the species *X. kroyeri* were published after 2000, adding important new information on the systematics of the genus, demonstrating the presence of cryptic species in the Atlantic and outlining the geographical distribution and the levels of genetic diversity and population structuring of Brazilian populations (GUSMÃO and SOLÉ-CAVA, 2002; VOLOCH and SOLÉ-CAVA, 2005; GUSMÃO *et al.,* 2006). The population boundaries were assessed based on allozyme markers, which are known to be less variable than other molecular markers currently used in shrimp genetic studies, such as the mitochondrial genes COI, CYTB and D-loop region, or microsatellites (HUNTER *et al.*, 2008; NAHAVANDI *et al.*, 2011; DUMONT *et al.*, 2009). Despite the great commercial importance of this resource, few efforts have been directed to characterize the genetic variability of populations and to clarify the boundaries of genetic stocks of the Atlantic *Xiphopenaeus* species.

Sequence polymorphisms of the mitochondrial COI gene have been used to define the geographic distribution of the *Xiphopenaeus* cryptic species (PIERGIORGE *et al.*, unpublished data), and also, the efforts of few Brazilian research groups have played an important role in the development of species-specific and heterologous microsatellite marker loci for the genetic characterization of the *Xiphopenaeus* species (FRANCISCO *et al.*, 2009; FREITAS *et al.*, 2007). However, few results have so far been generated using such markers.

Recently, geometric morphometrics has emerged as a powerful methodology for identifying operational taxonomic units, corroborating genetic data with great similarity of results (CHIARI *et al.*, 2009; FRANCOY and FONSECA, 2010), thus representing a promising tool to assist the identification of genetic stocks.

Geometric morphometrics has been recently used to characterize *Xiphopenaeus* populations from the south and southeastern coasts of Brazil (PIERGIORGE *et al*., unpublished data).

This study has the main objective of gathering the information produced so far about molecular systematics, patterns of geographic distribution of cryptic *Xiphopenaeus* species, genetic and morphometric characterization of variability and delineation of population boundaries. These were generated using different methodologies, including published as well as unpublished data, in order to determine consensus patterns of genetic variability distribution and structuring, aiming to support management and conservation actions, and mainly to identify gaps of knowledge and direct future efforts to address them.

CLASSIFICATION OF *Xiphopenaeus* SMITH, 1869

Morphological description of the genus

The genus *Xiphopenaeus* can be distinguished among the other Penaeidae genera by the following characters: rostrum armed with dorsal teeth only; first segment of antennular peduncle lacking parapenaeid spine; carapace with antennal and hepatic spines present; fourth and fifth pereopods elongate, much longer than the third one, with multiarticulate dactyl; all pereopods with exopods; sixth abdominal somite bearing interrupted cicatrix; telson unarmed.

Historical classification

Xiphopenaeus kroyeri, the only currently valid species of the genus, was first described based on a specimen from Rio de Janeiro, as part of the genus *Penaeus* (*P. kroyeri* Heller, 1862). The genus *Xiphopenaeus* was erected by SMITH only in 1969 (SMITH, 1969). In the same paper, a new species, *X. hartii*, was described, based on a sample obtained in Caravelas, Bahia. Later, *X. hartii* Smith, 1969, was considered a junior synonymous of *P. kroyeri,* and also a new combination to *Xiphopenaeus* genus took place, so that, until 1907, the genus *Xiphopenaeus* had only one species, *X. kroyeri* (Heller, 1862). BOUVIER (1907) described a new species, *X. riveti*, from Paita, Peru, in the Southeastern Pacific coast.

Until 1997, *Xiphopenaeus* was considered as having two species: one from the Atlantic coast, *X. kroyeri,* and the other from the Pacific coast, *X. riveti*. Morphological similarities were largely noticed, as in many American species present on both sides of the continent, which were only divided by the establishment of the Isthmus of Panama, during the Pleistocene (DALL *et al*., 1990). Therefore, in a taxonomic review of penaeidean shrimp genera, *X. riveti* was considered another junior synonymous of *X. kroyeri* (PÉREZ-FARFANTE and KENSLEY, 1997). The authors justified this change by the lack of significant morphological variations between the Atlantic and Pacific specimens. Consequently, *Xiphopenaeus* was considered a monotypic genus, and the distribution of *X. kroyeri* was enlarged, extending into the Atlantic Ocean from North Carolina (United States) to Florianópolis (Brazil), and in the Pacific Ocean from Sinamoa (Mexico) to Paita (Peru).

MOLECULAR SYSTEMATICS

Molecular identification of cryptic species

In 2002, species-specific PCR-RFLP markers, based on partial sequences of the mitochondrial cytochrome oxidase I gene, were developed for accurate identification of seven commercial Brazilian species (GUSMÃO and SOLÉ-CAVA 2002). Except for *X. kroyeri*, all the species presented unique electrophoretic patterns after endonuclease digestions. *Xiphopenaeus* samples from Natal (RN), Nova Almeida (ES) and Arraial do Cabo (RJ), however, revealed two different haplotypes: one, exclusive from the northeastern population of Natal, and a second from the two southwestern populations of Nova Almeida and Arraial do Cabo. These preliminary results indicated the occurrence of genetic heterogeneity among *X. kroyeri* populations distributed along the Brazilian coastline.

The presence of marked genetic differences among *X. kroyeri* populations from southeastern Brazil was later reported by VOLOCH and SOLÉ-CAVA (2005), who used ten allozyme *loci* for the investigation of genetic structuring patterns. Significant differences (F_{ST} = 0.223) were detected between the population from Ubatuba (SP) and the two populations from Nova Almeida and

Cabo Frio, which formed a single panmitic unit (VOLOCH and SOLÉ-CAVA, 2005). The population from Ubatuba, however, presented significant departures from Hardy-Weinberg expectations in two loci, due to strong deficiencies in observed heterozygosities (for *Ldh*2: H_o = 0.0). These observations led the authors to suggest that further investigations could be made to clarify the observed patterns of genotypic frequencies.

These two previous studies served as starting point for a broader investigation, where 174 samples were analyzed from Natal, Poças, Nova Almeida, Cabo Frio and Ubatuba, combining analyses of allozymes, PCR/RFLP and sequence polymorphisms of the mitochondrial COI gene (GUSMÃO *et al.*, 2006). From a total of fifteen loci analyzed, five diagnostic loci were observed in both Natal and Ubatuba populations, including the previously studied *Ldh*2 locus (VOLOCH and SOLÉ-CAVA, 2005). As a consequence, strong heterozygote deficiencies (F_{IS} = 0.575; P<0.05) were also detected in the population of Ubatuba and also in Natal. These deficiencies were probably due to the presence of fixed alternative alleles in these five loci (*Ldh*-2, *Mpi*, *Pep*-2, *Pgm*-1, *Pgm*-2), in the same individuals within each population, indicating the existence of two different species for the sea-bob shrimp of Natal and Ubatuba. These results were consistent with those formerly observed in the two previous studies.

Additionally, the results of PCR/RFLP and sequencing analysis of the COI gene, in samples from different populations from the Atlantic and the Pacific, confirmed the presence of two cryptic *Xiphopenaeus* species in the Atlantic, referred to as *Xiphopenaeus* sp. 1 and sp. 2, and indicated the occurrence of a third species from the Pacific, considered *X. riveti* Bouvier (1907) (GUSMÃO *et al.*, 2006). Partial sequencing of the COI gene showed high levels of genetic divergence among species of the Atlantic (12%), despite the great morphological similarity. The two species of the Atlantic exhibited different distributions and abundances along the coast, but the breadth of the geographical distribution of the two species was not completely elucidated.

*Geographical occurrence of the Atlantic **Xiphopenaeus** cryptic species*

Xiphopenaeus kroyeri (*sensu* PÉREZ-FARFANTE and KENSLEY, 1997), on the west coast of the Atlantic Ocean, ranges from North Carolina, in the United States, to Rio Grande do Sul, Brazil (SANTOS and IVO, 2000).

According to GUSMÃO *et al*. (2006), *Xiphopenaeus* sp. 1 was found in all localities, from Ubatuba (São Paulo, Brazil) to Caracas (Venezuela), and seemed to have a continuous distribution along the southwest coast of the Atlantic, while *Xiphopenaeus* sp. 2 was only observed in Natal and Ubatuba, signaling a disjoint distribution pattern. The occurrence of a discrete distribution pattern of *Xiphopenaeus* sp. 2 could also be explained by other factors, such as seasonal changes in the composition of species, or differences in species abundance among locations, requiring further studies to be fully comprehended.

Recently, new genetic studies of Brazilian *Xiphopenaeus* populations using identification methodologies based on the differences described by GUSMÃO *et al.* (2006) corroborated the previous evidence on the distribution patterns of the cryptic species. In 2009, a new point of occurrence, Cananéia, was observed for *Xiphopenaeus* sp. 2 along the coast of São Paulo (FRANCISCO, 2009). Afterwards, an extensive sampling was done in northeast, southeast and southern Brazil, aiming at mapping the occurrence of the two cryptic species along the coast by means of PCR/RFLP of 363 *Xiphopenaeus* samples, and sequencing of 74 specimens from nine localities (PIERGIORGE *et al.,* unpublished data). The map in Figure 1 summarizes the currently known distribution for these cryptic species in the southwest Atlantic. One consensus COI sequence from GenBank was also included herein, corresponding to haplotype "A" from *Xiphopenaeus* sp. 1; this consensus sequence was obtained from four individual samples from two different localities, Guaratuba (SP) and São Luis do Maranhão (Rodrigo MAGGIONI, personal communication, 2012; GenBank Accession nº AY135200.1), also included in the map.

As previously discussed by GUSMÃO *et al.* (2006), the discrete distribution pattern observed for *Xiphopenaeus* sp. 2 may be due to historical or current factors; among the latter, the population reduction due to over-fishing can be a determinant of the disjoint distribution. Regardless of the reasons that shaped present patterns of distribution, the occurrence of a species with limited distribution and difficult to capture, coupled with the current situation of overfishing of the sea-bob shrimp stocks in the south-southeast coast of Brazil, suggests that the current populations might be the remnants of a larger population. These indicators point to the necessity that effective measures are taken to ensure that these stocks are not eroded locally in their regions of occurrence, in order to conserve the genetic diversity of populations and guarantee resource sustainability in medium and long term.

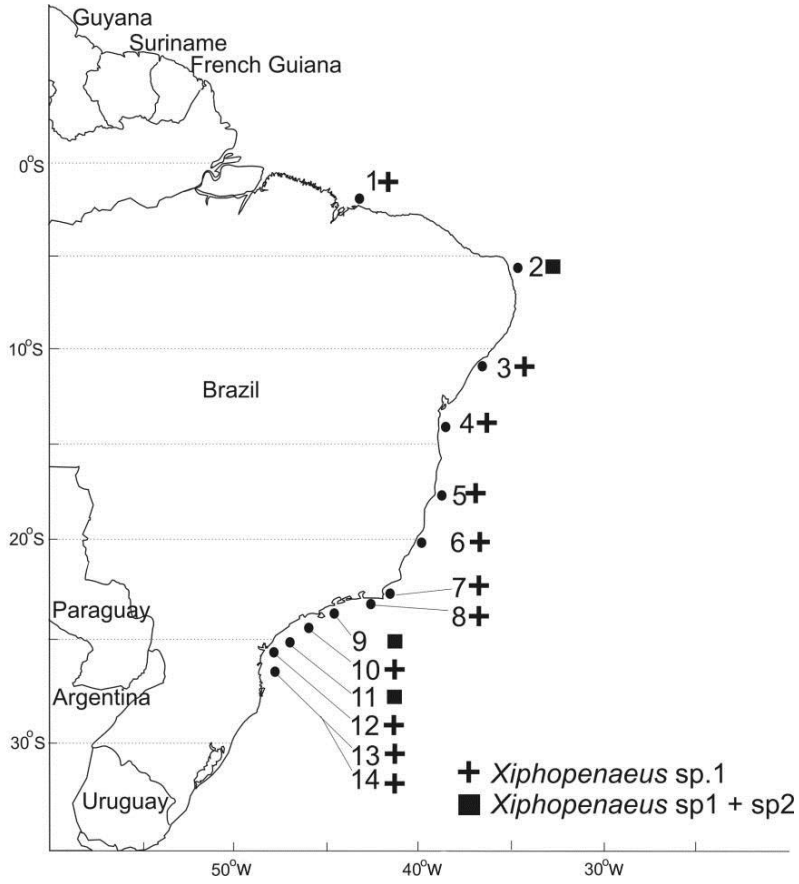

Figure 1. Geographical occurrence of the Atlantic *Xiphopenaeus* cryptic species. Locality numbers: 1- São Luis (1° 59' 56.7816" S, 44° 19' 7.8528" W); 2- Natal (5°52'S, 35°10'W); 3- Poças (11°46'S, 37°32W); 4- Ilhéus (14°46'S, 39°01'W); 5- Caravelas (17°44'S, 39°15'W); 6- Nova Almeida (20°03'S, 40°11'W); 7- Atafona (40°11'W, 41°0'W); 8- Arraial do Cabo (22°58'S, 42°01'W); 9- Ubatuba (23°26'S, 45°04'W); 10- Santos (23°58'S, 46° 19'W); 11- Cananéia (25°02'S, 47°55'W); 12- Guaratuba (25° 52' 44.3778" S, 48° 31' 35.7996" W); 13- Barra Velha (26°37'S, 48°40'); 14- Balneário Camboriú (26°59'07"S, 48°35'58"W).

Molecular methods for identification of cryptic species

The precise identification of commercial species is a prerequisite for the effective management of fisheries. In situations where the morphological similarity between species is large, as in the case of sibling sea-bob shrimp species, identification errors can be frequent. The misidentification of fishery resources can lead to misinterpretation of catch data in areas where there is overlap of species, leading to inadequate fisheries management measures, which can contribute to the depletion of populations

(WARD, 2000). The correct identification of species is also fundamental to the supervision of fishing activities, as well as for monitoring the trade and export of fresh or industrialized fish (GUSMÃO and SOLÉ-CAVA, 2002). The use of genetic tools in fisheries management enables the unambiguous identification of morphologically similar species, through the use of species-specific molecular markers. Species-specific markers for precise identification of the cryptic *Xiphopenaeus* species based on two different methodologies are described hereafter.

a) Allozymes

Although the use of allozymes has fallen into disuse with the advent of molecular markers based on the direct analysis of DNA polymorphisms, allozyme analysis represents an effective and cheap methodology for the discrimination of similar species (CHUNG and CHUNG, 2012), which has the advantage of using universal protocols (SCHLÖTTERER, 2004).

Allozyme markers for the identification of specimens from the two Atlantic *Xiphopenaeus* species were developed (GUSMÃO *et al.*, 2006). Despite the fact that five diagnostic loci were observed in sympatry, with alternative alleles fixed in the two species of populations from Natal and Ubatuba, the best allozyme markers for the identification of the cryptic species of the southwest Atlantic are enzymes *Ldh*-2 and *Pgm*-2, which have different alleles (not shared) across species distributions along the Brazilian coast in all studied populations so far (Table 1). Analyses can be conducted using horizontal 12.5 % starch gel electrophoresis and standard methodology (see MURPHY *et al.*, 1990; GUSMÃO *et al.*, 2006).

b) PCR/RFLP of the mitochondrial COI gene

Restriction endonucleases (restriction enzymes) are capable of cleaving double-stranded DNA in specific recognition sequences of small size (restriction sites). Genetic polymorphisms in the recognition sites are detected by the size separation of DNA or PCR fragments by electrophoresis in agarose or polyacrylamide (see KAPUSCINSKI and MILLER, 2007).

Table 1. Allozyme diagnostic *loci* and buffer systems used for identification of Atlantic *Xiphopenaeus* species (GUSMÃO *et al.*, 2006). Enzyme Commission numbers (E.C.) and abbreviations; (+) allele presence; (-) allele absence; * Buffer systems: TEM = 0.10 M Tris, 0.01 M EDTA, 0.10 M maleate, pH 7.4 (BREWER, 1970); TC7 = 0.135 M Tris, 0.043 M citrate, pH 7.0 (SHAW and PRASAD, 1970).

Enzyme	EC	Abbreviation	Locus/ (alleles)	Fixed alleles sp. 1	Fixed alleles sp. 2	Buffer*
			Ldh-2			
Lactate dehydrogenase	1.1.1.27	LDH	(A)	-	+	TEM
			(B)	+	-	
			Pgm-2			
Phosphoglucomutase	5.4.2.2	PGM	(A)	+	-	TC7
			(B)	-	+	
			(C)	-	+	

In 2002, a molecular diagnostic system based on the PCR amplification of partial cytochrome oxidase I gene and cleavage of the amplified product with *Hinf* I and *Hinc* II endonucleases was designed for the identification of seven Brazilian commercial penaeid shrimp species, including *X. kroyeri* (*sensu* PÉREZ-FARFANTE and KENSLEY, 1997). The combined endonuclease cleavage patterns of the amplified product (PCR-RFLP) generated species-specific unique patterns for all species, except for *X. kroyeri*. Although there have been two different combined cleavage patterns (haplotypes) recognized for the populations of *X. kroyeri*, one (haplotype *Hinf*I/*Hinc*II = E/C) from the northeast (Natal), and the other (haplotype *Hinf*I/*Hinc*II = A/D) from southeast of Brazil (Rio de Janeiro and Espírito Santo), the two sibling species have not been detected yet and were considered as one. Later, it was found that each of the two haplotypes represented, in fact, one of the two species occurring in Brazil (GUSMÃO *et al.*, 2006).

The haplotype A/D corresponded to *Xiphopenaeus* sp. 1 and the second haplotype, E/C, to sp. 2. Since 2002, new COI sequences have been described for the two species (GUSMÃO *et al.*, 2006; MAGGIONI; GenBank Accession Nº AY135200.1; PIERGIORGE *et al.*, unpublished data). However, despite the variability observed in sequences from the same region of the COI gene, we observed that the endonuclease recognition sites used by GUSMÃO and SOLÉ-CAVA (2002) for the recognition of alternative species are conserved within each species, which still keeps the system effective for the identification of *Xiphopenaeus* spp.

The methodology for identification of cryptic *Xiphopenaeus* species using COI PCR-RFLP analysis can be conducted using the conditions described in the original article of GUSMÃO and SOLÉ-CAVA (2002), but interpreting the two different *X. kroyeri* haplotypes as belonging to the alternative species (Figure 2).

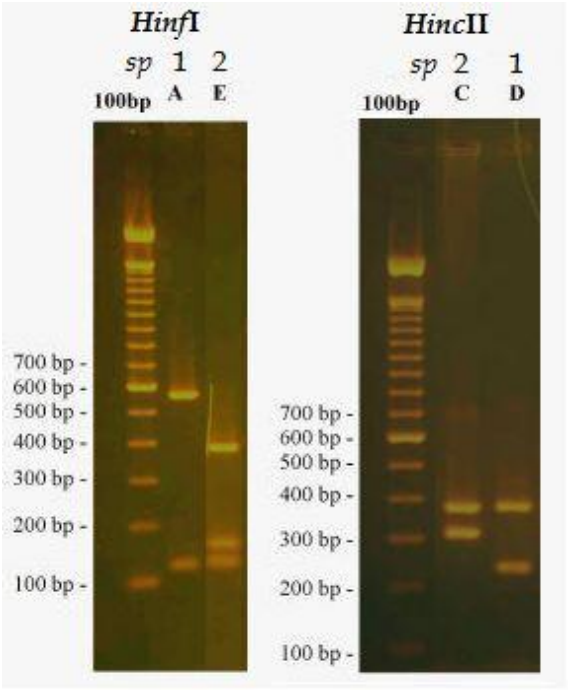

Figure 2. Species-specific electrophoretic patterns for the two cryptic *Xiphopenaeus* species, after digestion of PCR products with *Hinf*I or *Hinc*II endonucleases. Modified from GUSMÃO and SOLÉ-CAVA (2002). *Hinf*I) Pattern "A" *Xiphopenaeus* sp. 1; pattern "E" *Xiphopenaeus* sp. 2; *Hinc*II) Pattern "D" *Xiphopenaeus* sp. 1; pattern "C" *Xiphopenaeus* sp. 2.

As only the restriction maps, not the COI sequences, were published by GUSMÃO and SOLÉ-CAVA (2002), here we present the consensus COI haplotypes for *Xiphopenaeus* sp. 1 and sp. 2, highlighting the respective cleavage sites for endonucleases *Hinf*I and *Hinc*II (Figure 3), based on previously published results (GUSMÃO and SOLÉ-CAVA, 2002; GUSMÃO *et al.*, 2006), and new results produced from PCR/RFLP identification of 338 *Xiphopenaeus* specimens (314 sp. 1 and 24 sp. 2), plus 20 new sequenced haplotypes (17 for sp. 1 and 3 for sp. 2), obtained in nine locations along the Brazilian coastline (PIERGIORGE *et al.*, unpublished data).

The phylogenetic reconstruction shown in Figure 4 was done using the same methodology employed by GUSMÃO *et al.* (2006). Thirty-six sequences were analyzed. From these, 16 were obtained from GenBank (NCBI, www.ncbi.nlm.nih.gov/), and 20 are new sequences from the same region of the COI gene (PIERGIORGE *et al.*, unpublished data). Despite the presence of new haplotypes for both species, three clades continue to form with high statistical support, the two Atlantic species, sp.1 and sp. 2, and the Pacific species *X. riveti*. In addition, like the result shown by GUSMÃO *et al.* (2006), the sister group of *Xiphopenaeus* sp. 2 remains the

Pacific species, X. *riveti*. Despite the high morphological resemblance, interspecific K2P genetic distances were high, varying from 0.120 to 0.125 (12%), whereas intraspecific distances ranged from 0 to 0.007 (0.7%) in sp. 1, from 0 to 0.002 (0.2%) in sp. 2, and from 0.002 to 0.005 (0.5%) in *X. riveti*.

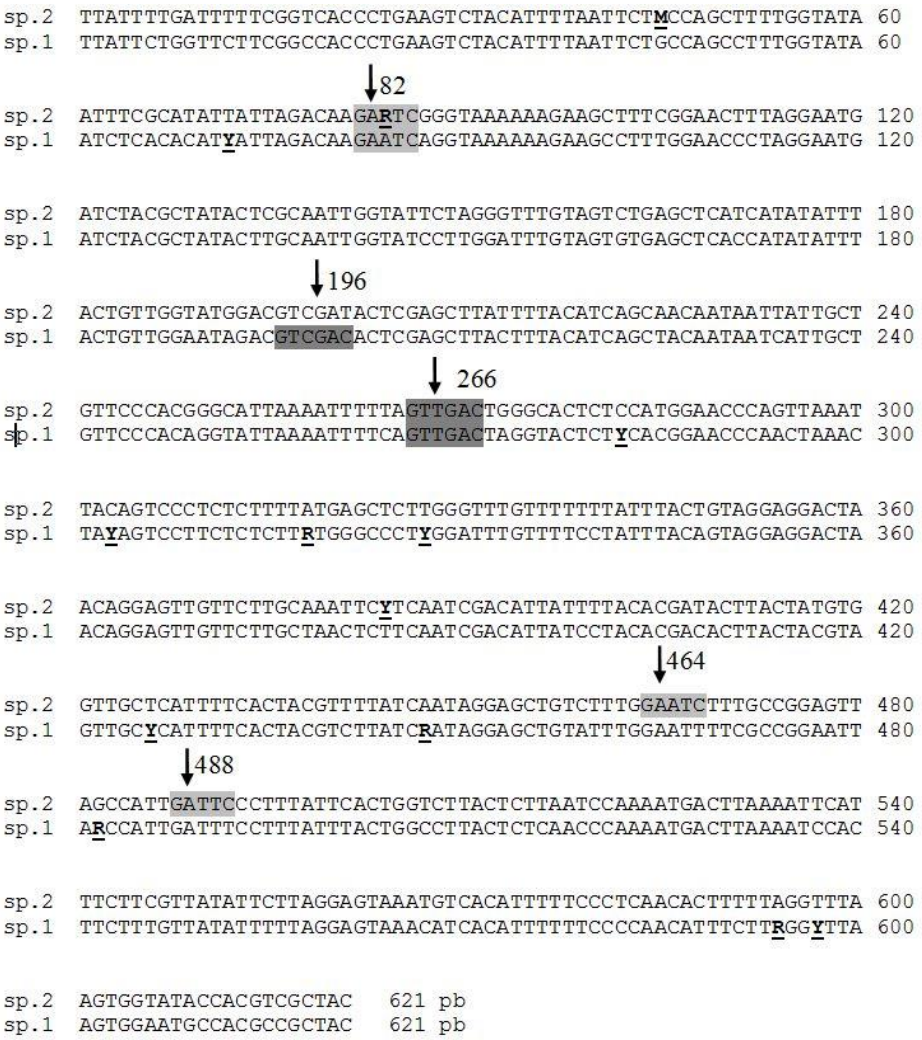

Figure 3. Sequence alignments of COI consensus sequences of *Xiphopenaeus* sp. 1 and sp.2, highlighting the restriction sites for *Hinf*I (GTPy↓PuAC) and *Hinc*II (G↓ANTC). Polymorphic sites: (R) A or G; (Y) C or T; (M) A or C. Numbers: site positions.

GENETIC DIVERSITY AND POPULATION STRUCTURING OF THE ATLANTIC *Xiphopenaeus* SPECIES

Genetic surveys

To date, only two studies were published in which different *Xiphopenaeus* populations were genetically characterized, both using allozymes. In the first study, three populations of southeastern Brazil, Ubatuba, Nova Almeida and Cabo Frio were analyzed using ten allozyme loci for the investigation of genetic structuring patterns (VOLOCH and SOLÉ-CAVA, 2005). As previously discussed, the "population" of Ubatuba that was analyzed as single evolutionary unit comprises a mixture of two species (GUSMÃO *et al.*, 2006). Thus, significant deviations from the Hardy-Weinberg equilibrium which were observed are probably a consequence of mixing genetic stocks. In contrast, Cabo Frio and Nova Almeida populations, where only *Xiphopenaeus* sp. 1 is observed, presented no significant departures

from Hardy-Weinberg expectations, and there were no significant differences between these populations, indicating the presence of a single genetic stock (VOLOCH and SOLÉ-CAVA, 2005). Of the ten loci studied, seven were polymorphic (70.0%). Mean observed and expected heterozygosities ranged from 0.111 to 0.116 and 0.13 to 0.126 respectively.

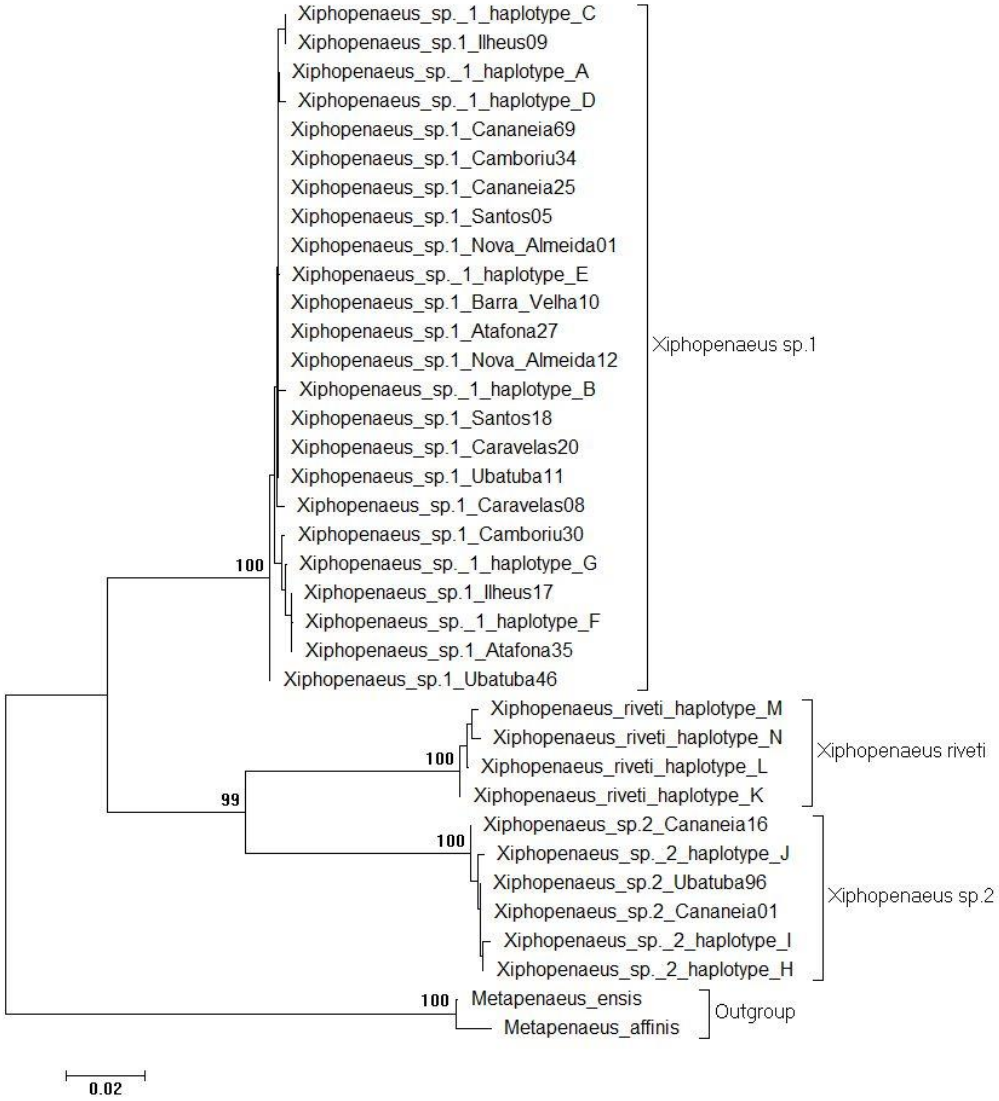

Figure 4. *Xiphopenaeus* spp. Cytochrome oxidase I neighbor-joining tree based on Kimura-2-parameter distances (KIMURA, 1980). Haplotypes (A-M) (GUSMÃO *et al.*, 2006; GenBank accession numbers DQ084367-DQ084380); Out-groups: *Metapenaeus affinis* (QUAN *et al.*, 2004; accession number AY264886), *Metapenaeus ensis* (LAVERY *et al.*, 2004; accession number AF279830); Remaining sequences (PIERGIORGE *et al.*, unpublished data); Numbers above branches are bootstrap values (1,000 replicates). Only values higher than 70 were shown.

The second study described five populations of *Xiphopenaeus* sp. 1, Natal, Poças, Nova Almeida, Cabo Frio and Ubatuba and two of *Xiphopenaeus* sp. 2, Natal and Ubatuba (GUSMÃO *et al.*, 2006). Of the 15 loci studied, 11 were polymorphic (73.3%) and mean observed (H_o) and expected (H_e) heterozygosities from 0.019 to 0.114 and from 0.045 to 0.118 respectively, in sp. 1, and from 0.101 to 0.105 and 0.092 to 0.095, respectively, in sp. 2. The heterozygosities levels reported in both

studies are within the range of variability normally observed in penaeid shrimp species (H_e = 0.007- 0.12; BENZIE, 2000; GUSMÃO *et al.*, 2005). Data analyses, considering the presence of two species, resulted in no within-population deviations from Hardy-Weinberg expectations. Significant statistical differences were detected among populations of *Xiphopenaeus* sp. 1 (F_{ST} = 0.020-0.122; P<0.05) and also between the two populations where *Xiphopenaeus* sp. 2 was observed (F_{ST} = 0.055; P<0.05), indicating the existence of genetically structured populations for both species along the Brazilian coast. *Xiphopenaeus* sp.1 population from Nova Almeida was significantly different from the two populations of Cabo Frio (F_{ST} = 0.020; P<0.05) and Poças (F_{ST} = 0.024; P<0.05), and significant differences were also found between Cabo Frio and Ubatuba (F_{ST} = 0.093; P<0.05), and between Poças and Ubatuba (F_{ST} = 0.122; P<0.05). Likewise, the populations of *Xiphopenaeus* sp. 2 from Natal and Ubatuba also comprise different genetic stocks (F_{ST} = 0.055; P<0.01). Although there were no significant differences between Nova Almeida and Arraial do Cabo in the earlier work by VOLOCH and SOLÉ-CAVA (2005), this may reflect the smaller number of loci analyzed in this study.

More recently, hyper variable microsatellite loci have also been developed for *Xiphopenaeus* species (FRANCISCO *et al.*, 2009). Several species-specific primers were designed to *X. kroyeri*, and other heterologous primers have been tested for the genus, resulting in positive amplifications (Table 2). However, to date, no studies have been published using these markers for population analyzes of *Xiphopenaeus* species.

Table 2. Microsatellite primers available in literature, produced for *X. kroyeri* (Xkro) or for other penaeid species (Fbra, Rcon, Lvan), but which produced positive results in cross-species amplification tests. Source: 1) FRANCISCO *et al.*, 2009; 2) FREITAS *et al.*, 2007.

Locus/GenBank nº	Primer sequences (5′-3′)	Motif	Source
Xkro 10/EU559719	F: GTGTTGAGACGAAACATGG R: CGACCTAGAGAATGTAGGCA	(TC)8	1
Xkro 11/EU559720	F: CGATAGCATCTACCTCTTCC R: ACAGAGGGTATTTCACTTCATC	(CTTCCT)9	1
Xkro 12/EU559721	F: TCTCGGTTTCTCTTGTCACT R: AGCTGGGACTTCTCCTTAC	(TCTG)6...(TCTG)7 (TC)6CT(TC)4	1
Xkro 13/EU559722	F: CACGCTCATATACACTCACA R: GAACTGCAGGTGGTGAAGTC	(CA)6	1
Fbra 01/EU559711	F: ACGCACACAGAGCACATAC R: CTTGTCTTGGAAACGTCCTA	(CA)10	1
Fbra 02/EU559712	F: ACTTTGTAATACGGAGTGCG R: TACATATTGGGAAACGAAGG	(GTCT)5...(CT)6	1
Fbra 03/EU559713	F: ACACACACGCACACACTTAT R: TAGCCTCTTTGTGGTTTGTT	(AC)7	1
Fbra 04/EU59714	F: CTTGTTTATGGTGGGATGAG R: CACAGACAGAGATTTATGCG	(TGTC)5...(TC)11	1
Rcon 06/EU59716	F: GTTGCTTATTGCTGAACC R: GACAACGCCGACTATAAC	(GTT)12	1
Rcon 07/EU59717	F: AATAGGATTCGGATACGC R: AGCAAAAAGTCTGCGTTC	(GTTT)5	1
Lvan 06/DQ988334	F: TAGATCCGTTTAAGTCACA R: GTATAATGTCGAATGCTCAC	(TATC)2 … (TGTC)3 … (TGTC)3	2
Lvan 08 /DQ988336	F: CTTCACAGAGGTTGGATAG R: CGATAAGGAAACTGACATTG	(AGC)8	2
Lvan 09 /DQ988337	F: ATCTCGATAAGGAAACTGAC R: GACAGGTTTGTCTTCACAG	(TGC)9	2
Lvan 10 /DQ988335	F: ATAAGAAGGTCGTTTCTCTC R: AATACGACTCAACTATAGGG	(A)4(AG)6(AAAG)3(A)6	2

Geometric Morphometrics analyses of ***Xiphopenaeus*** *sp. 1 populations*

The partial and relative warps (BOOKSTEIN, 1991) of geometric morphometrics methodology have proven effective in differentiating operational taxonomic units (FRANCOY and FONSECA, 2010). These warps tests are based on the use of landmarks (ROHLF and MARCUS, 1993) which allow the identification of variations in shape among the morphological homologous structures of the different specimens analyzed. The results obtained with geometric morphometrics can be compared with genetic studies, with great similarity (CHIARI *et al.*, 2009; FRANCOY and FONSECA, 2010).

Different Brazilian populations of *X. kroyeri* sp. 1 are being analyzed using geometric morphometrics (PIERGIORGE *et al.*, unpublished data). For this study we choose to use only carapace characters due to precise collection of measurements on this structure and its complexity as a morphological trait, probably a result of the interaction of many genes. A Total of 21 landmarks on the carapace were selected (Figure 5).

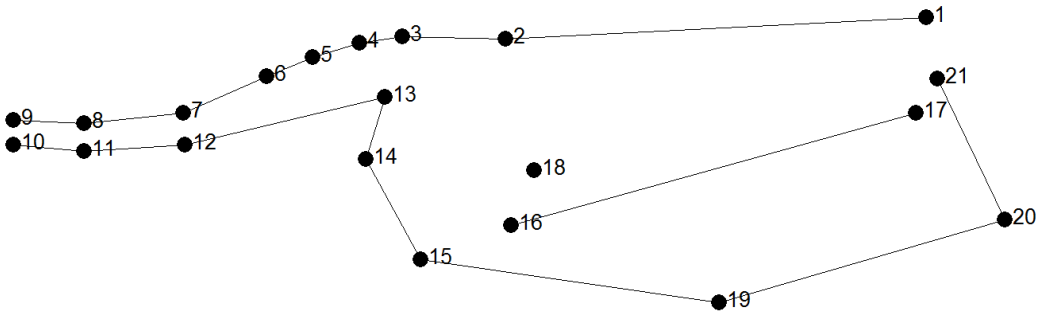

Figure 5. Consensus of all 21 landmarks selected for the analysis.

Preliminary results can be reported observing specimens of both sexes from four locations on the Brazilian coast: Nova Almeida, Atafona, Ubatuba and Balneário Camboriú (see Figure 1). According to the statistical analysis performed (Multivariate Analysis of Variance, MANOVA; CRAWLEY, 2007), all these four populations are significantly different from one another (Table 3). Significant differences are found also between sexes. However, there is no interaction between sex and location affecting *X. kroyeri* specimens.

Other results can be also extracted from preliminary analyses. Performing a Linear Discriminant Analysis (LDA; CRAWLEY, 2007) (Figure 6), the four populations remain clearly separated from each other. Examining LD1/LD2 graphics individually, Nova Almeida and Atafona populations are closer to each other and this was also observed for Balneário Camboriú and São Paulo. The same pattern is present in the LD2/LD3 graphic, but here Nova Almeida and Atafona are even closer populations. This pattern changes only in the LD1/LD3 graphic, where Nova Almeida is completely isolated from the other three, Atafona and Balneário Camboriú closer to each other, and Ubatuba stays between the two "groups". However, more than 80% of the variation is found within LD1 (0.4615) and LD2 (0.3836), so there is more confidence in the LD1/LD2 graphic. These results can be compared with those obtained using allozymes (GUSMÃO *et al.*, 2006), although, in the later, there are two locations not analyzed herein: Poças (Bahia) and Cabo Frio (Rio de Janeiro). On the other hand, two populations which were analyzed by geometric morphometrics did not figure in the work of GUSMÃO *et al.* (2006): Atafona, which is located north of the State of Rio de Janeiro, and Camboriú.

Table 3. Manova results. Df = Degrees of freedom; F = Fisher's F-test; num = numerator; den = denominator; Pr = p-value. Significance codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1.

	Df	Pillai	Approx. F	numDf	denDf	Pr (>F)
Local	1	0.238709	8.0741	4	103	1.056e-05 ***
Sex	1	0.216835	7.1294	4	103	4.156e-05 ***
Local:sex	1	0.037432	1.0014	4	103	0.4104
Residuals	106					

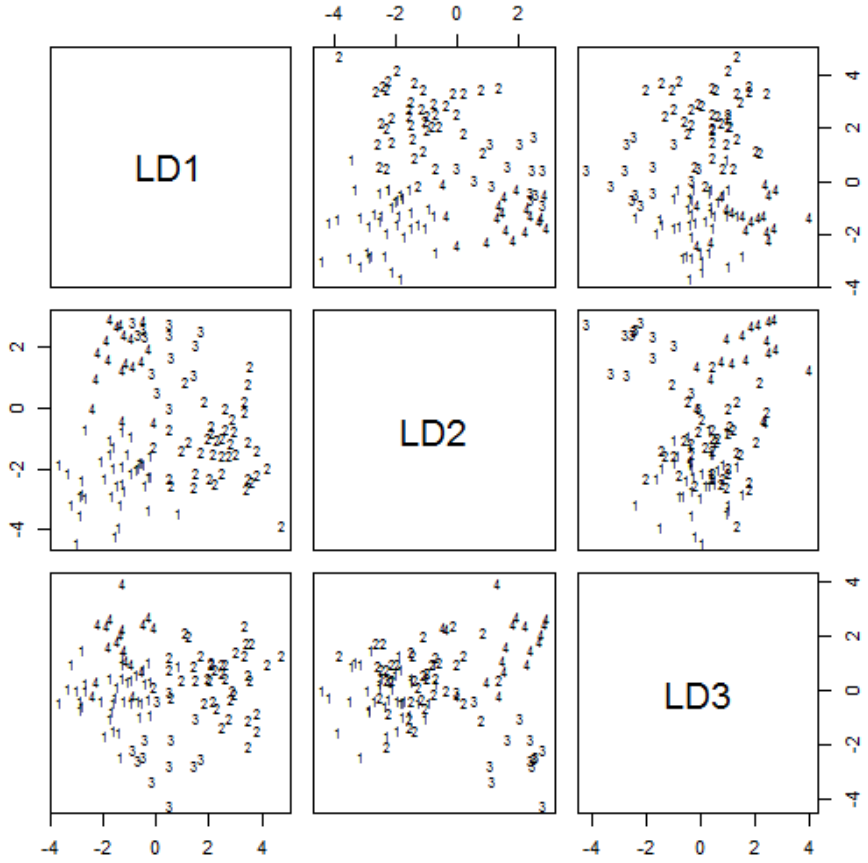

Figure 6. Discriminant analysis. The numbers represent each of the four populations (1- Atafona; 2 – Nova Almeida; 3 – Ubatuba; 4 – Balneário Camboriu).

Allozyme population analyses resulted in significant F_{ST} values, indicating that *Xiphopenaeus* sp. 1 samples are genetically structured along the studied area (Nova Almeida ≠ Poças and Cabo Frio; and Cabo Frio ≠ Ubatuba). In the morphological results, presented in the LD1/LD2 graphic, as in the allozyme results, Nova Almeida was significantly different from the population from Rio de Janeiro (Atafona instead of Cabo Frio), and, in both studies, significant differences were also found between the population from Rio de Janeiro (Atafona or Cabo Frio) and Ubatuba. The combined results show that *Xiphopenaeus* sp. 1 populations from the southeastern coast (Espírito Santo, Rio de Janeiro and São Paulo) comprise different genetic stocks along the coast.

Examining the specimens morphology through the graphics obtained in TPS programs (Figure 7) it is possible to notice that there are some very subtle variations, as in rostrum

curvature (landmarks 3-12), on the position of hepatic spine (landmark 18) and the length of the sub marginal carina (landmarks 19-20). However, with the significant variations found in morphometrics, we can conclude that *X. kroyeri* sp. 1 populations are structured along the studied area, comprising Nova Almeida, Atafona, Ubatuba and Balneário Camboriú, and maybe they constitute different fishery stocks along the Brazilian coast.

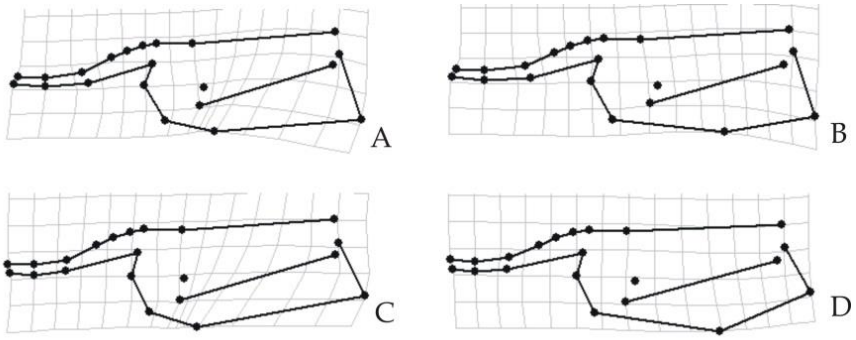

Figure 7. Relative warps of the four locations: a) Nova Almeida; b) Atafona; c) Ubatuba; d) Balneário Camboriú.

FINAL CONSIDERATIONS

The majority of the genetic studies focused attention on populations from the southeastern and south of Brazil, so that there is an enormous gap of information regarding the populations from the northeastern and principally northern coast. The more comprehensive results were produced from analyzes of the geographic distribution of cryptic species of the Atlantic, including samples from the northeast, southeast and southern Brazil, from São Luís do Maranhão (1°59'56.7816"S, 44° 19'7.8528"W) to Balneário Camboriú (–26º59'07"S, –48º35'58"W). The available data on the variability and genetic structure of Brazilian populations come from studies of allozyme and RFLP, or sequencing of the mitochondrial COI gene, which are slightly polymorphic markers in comparison with other genetic markers used in population studies, such as microsatellite loci that could reveal more subtle patterns of population structure, migration rates and population history. Although several microsatellite loci have already been developed for the sea-bob shrimp, population analyzes using such markers have not yet been published.

Despite the advantages of the use of molecular approaches, in terms of providing more or less neutral markers and greater levels of polymorphisms than the morphological characteristics previously used in genetic studies, direct observation of the morphology is still an essential component in genetic and ecological studies of populations (RUMPUNEN and BARTISH, 2002), and thus, also on the determination of genetic fisheries stocks. The genetic structure of populations, caused by restricted gene flow, random genetic drift, new mutations and natural selection of sub-populations in different environments may be revealed as variations in color and local adaptations on morphological features that indicate populational divergences (SPREITZER *et al*., 2012). Besides, genetic and environmental differences can cause diversity in ontogenetic development, resulting in body size variation (SILVA *et al*., 2008). Even among populations subjected to slightly differentiated environmental conditions, morphological differences may occur as a result of evolutionary processes other than local selection. These can also be detected using geometric morphometrics (SPREITZER *et al*., 2012). For these reasons, when molecular markers and morphological data are used in combination on the same populations, more conclusive information on the genetic structure can be obtained. In this sense, geometric morphometrics has proved to be an extremely useful tool in detecting subtle but significant differences among populations, corroborating genetic data based on

molecular markers. It has the ability to reveal fine-scale morphological differences in overall body shape (ADAMS *et al*., 2004; MADERBACHER *et al*., 2008). The combined results of allozymes and geometric morphometrics of *Xiphopenaeus* sp. 1 populations presented here, indicate that populations from the northeast, southeast and south coasts of Brazil comprise different genetic stocks.

Given the wide distribution of the sea-bob shrimp on the west coast of the Atlantic and taken into consideration its great economic importance, especially in southeastern Brazil, where the resource is overexploited, and where the two sibling species occur with overlapping stocks in certain locations, the paucity of studies on the genetics of *Xiphopenaeus* species is impressive. The available data, however, represent outstanding contributions to the systematics, delineation of the geographical distribution of cryptic species, and for the comprehension of the genetic structure of *Xiphopenaeus* spp. populations along the coast. This information combined with the methodologies available for molecular identification of the sibling species, can be used to support the outlining of effective measures for management and conservation of *Xiphopenaeus* fishery stocks, but greater efforts must be made to supplement current knowledge.

ACKNOWLEDGEMENTS

The authors would like to thank N.M.N. Abreu, A. P. Vidotto-Magnoni and, I. F. Machado for help with collecting samples, K.A. Morelli, for help with sequencing reactions, F.B. Pitombo by confection of the map of Figure 1 and Elisa Hoeschl by careful correction of the English.

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