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RESEARCH ARTICLE

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# Cryptic species and population structuring of the Atlantic and Pacific seabob shrimp species, *Xiphopenaeus kroyeri* and *Xiphopenaeus riveti*

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**Abstract** Seabob shrimps of the genus *Xiphopenaeus* are important fishery resources along the Atlantic and Pacific coasts of Central and South America. The genus was considered to comprise two species: the Atlantic *Xiphopenaeus kroyeri* (Heller, Sitzungsber Math Naturwiss cl kaiserliche Akad Wiss Wien 45:389–426, 1862), and the Pacific *Xiphopenaeus riveti* (Bouvier, Bull Mus Hist Nat Paris 13:113–116, 1907). In a recent review, *Xiphopenaeus* was regarded as a monotypic genus, on the basis that no clear morphological differences could be found between Pacific and Atlantic specimens (Pérez Farfante and Kensley, Mem Mus Nat Hist Nat Paris 175:1–79, 1997). In the present work, nuclear (allozymes), and mitochondrial (Cytochrome Oxidase I) genes were used to demonstrate the validity of *X. riveti* and reveal the presence of two cryptic species of *Xiphopenaeus* within *X. kroyeri* in the Atlantic Ocean. The high levels of molecular divergence among these species contrast with their high morphological resemblance. Interspecific sequence divergences (Kimura 2-parameter distance) varied from 0.106 to 0.151, whereas intraspecific distances ranged from 0 to 0.008 in *Xiphopenaeus* sp. 1, from 0 to 0.003 in *Xiphopenaeus* sp. 2, and from 0.002 to 0.005 in *X. riveti*. In addition, five diagnostic allozyme loci were found between sympatric samples of *Xiphopenaeus* sp. 1 and 2 along the Brazilian coast. The results suggest that *Xiphopenaeus* sp. 2 from the Atlantic is more closely related to the Pacific *X. riveti* than to the Atlantic *Xiphopenaeus* sp. 1. Furthermore, a high level of genetic structuring (*Xiphopenaeus* sp. 1: $F_{ST}=0.026$; $P<0.05$; *Xiphopenaeus* sp. 2: $F_{ST}=0.055$; $P<0.01$) was found in the Brazilian *Xiphopenaeus* populations, indicating the presence of different genetic stocks in both Atlantic species. These findings have important commercial implications as they show that the fisheries of the two Atlantic species must be managed separately, and that each one is comprised of different populations.

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## Introduction

Penaeid shrimp species are important economic resources in many countries (Sunden and Davis 1991). In the West Atlantic, the seabob, *Xiphopenaeus kroyeri* (Heller 1862) is one of the most common native shrimp species. In Guyana, Suriname, and the East coast of the United States, the average production of *X. kroyeri* between 1999 and 2003 was 17,500, 9,600, and 3,200 tons/year, respectively (FAO 2005). In Brazil, the average production of *X. kroyeri* in the same period was 11,500 tons/year, higher than the 7,400 tons/year of *Farfantepenaeus brasiliensis* and close to the 12,000 tons/year for the sum of all other native *Penaeus* (*sensu lato* Fabricius 1798) species (FAO 2005).

The genus *Xiphopenaeus* Smith 1869 was considered to be comprised of two species: the Atlantic *X. kroyeri*, and the Pacific *Xiphopenaeus riveti* (Bouvier 1907). However, in a recent review, *X. riveti* was considered to be a junior synonym of *X. kroyeri* based on lack of significant morphological differences between the Pacific and Atlantic specimens, thus making *Xiphopenaeus*

monotypic (Pérez Farfante and Kensley 1997). As a consequence of the synonymy, the distribution of *X. kroyeri* now ranges from North Carolina (USA) to Florianópolis (Brazil) in the Atlantic, and from Sinamoa (Mexico) to Paita (Peru), in the Pacific (Fig. 1; Pérez Farfante and Kensley 1997).

In a recent attempt to develop a molecular diagnostic system to identify different Brazilian commercial shrimp species, two different PCR/RFLP haplotypes of the cytochrome oxidase I (*COI*) mtDNA gene were found within populations of *X. kroyeri* from Northeast Brazil (Gusmão and Solé-Cava 2002). Moreover, a recent allozyme study of *X. kroyeri* from Southeast Brazil found very large heterozygote deficiencies (Voloch and Solé-Cava 2005). In order to verify if those deficiencies, as well as the PCR/RFLP differences, could result from the existence of cryptic species, as already observed in other Atlantic penaeid species (Gusmão et al. 2000), we used allozymes and *COI* sequences to compare samples of several *X. kroyeri* populations from both the Atlantic and Pacific coasts of Latin America.

DNA sequencing of the observed *COI* haplotypes, and allozyme analyses reveal the presence of two different cryptic *Xiphopenaeus* species in the Atlantic. *COI* sequencing also demonstrates that the Pacific *X. riveti* is a valid species. The large genetic divergence found among the three species contrasts with their high morphological resemblance. These findings have important implications for clarifying the taxonomic status of Atlantic and Pacific *Xiphopenaeus* spp.

## Materials and methods

### Collection of samples

For allozyme analyses, 176 individuals of *X. kroyeri* were obtained directly from fishermen straight after disembarkation of small trawlers, from five different locations along the Brazilian coast (Natal 5°52′S/35°10′W; Poças 11°46′S/37°32′W; Nova Almeida 20°03′S/40°11′W; Arraial do Cabo 22°58′S/42°01′W; and Ubatuba 23°26′S/45°04′W; Fig. 1). The samples were collected between February and August 2001, and cover about 2,500 km distance and a comprehensive range of the species distribution along the Brazilian coast. The samples were transported on dry ice to the laboratory, where they were morphologically identified per classification system of Pérez Farfante and Kensley (1997). A sample of muscle tissue of each individual was stored in liquid nitrogen until required for allozyme studies or DNA purification.

Twelve individuals from Caravelas (Bahia 17°44′S/39°15′W), collected in January 2005, were used for haplotype PCR/RFLP scoring, and DNA sequencing of a part of the *COI* gene of three of those specimens was


**Fig. 1** *Xiphopenaeus kroyeri* sampling sites. Gray area: putative geographic distribution of *X. kroyeri* (*sensu* Pérez Farfante and Kensley 1997)

performed. Four *X. kroyeri* individuals collected in Panama City (Pacific Ocean) (8°53′N/79°35′W; Fig. 1) and three collected in Caracas (Venezuela 10°36′N/66°59′W; Fig. 1), in July and November 2004 respectively, were used for *COI* sequencing. Total DNA extractions were performed using a modified CTAB protocol (Damato and Corach 1994; Gusmão and Solé-Cava 2002).

## Allozyme analyses

Analyses were conducted using horizontal 12.5% starch gel electrophoresis and standard methodology (Murphy et al. 1990; Gusmão et al. 2000). Combinations of the buffer and enzyme systems are shown in Table 1. Allozyme patterns were revealed using standard enzyme stains (Manchenko 1994). Genotype frequencies were used to estimate gene frequencies, heterozygosities, and unbiased genetic identities and distances (*I* and *D*; Nei 1978). Genetic identities were used to construct a UPGMA tree (Sneath and Sokal 1973). These data were analysed using the program BIOSYS Version 1.7 (Swofford and Selander 1981). Node support of the UPGMA tree was estimated through bootstrap pseudo-replication (Efron 1981; Felsenstein 1985) with the DISPAN program (Ota 1993). *F*-statistics were estimated according to Weir and Cockerham (1984). The significance of $F_{IS}$ ($H_o$: $F_{IS}=0$) and $F_{ST}$ ($H_o$: $F_{ST}=0$) were tested according to Waples (1987), using a permutation approach (with 1,000 replicates). A factorial correspondence analysis (FCA) was used to better analyze the genetic relationships among the different populations. This methodology uses the allelic data to project individuals into a multidimensional graphic, with each allele considered as an independent variable. These analyses were done with the program GENETIX Version 4.05 (Belkhir et al. 2004).

## PCR amplification and sequencing of partial COI gene

A 677 bp section of the 3′ end of the mitochondrial cytochrome oxidase subunit I gene was amplified using primers *COI*f [5′-CCT GCA GGA GGA GGA GA(C/T) CC-3′] (Palumbi and Benzie 1991) and CO10 [5′-TAA GCG TCT GGG TAG TCT GA(A/G) TA(T/G) CG-3′] (Baldwin et al. 1998). PCR reactions were performed in a PTC-100 Mini-cycler (MJ Research) programmed for one denaturation step at 94°C for 3 min, followed by 40 cycles at 94°C for 1 min, 51°C for 1 min, and 72°C for 45 s, and a final 5 min extension step at 72°C, as per Gusmão and Solé-Cava (2002). Negative controls, consisting of template-free reactions, were included in all PCR amplifications. Before purification, 5 µl of each PCR reaction were used for endonuclease cleavage for scoring composite *Hinf* I/*Hinc* II haplotypes, applying the previously developed PCR/RFLP diagnostic system (Gusmão and Solé-Cava 2002).

DNA sequencing was carried out using standard procedures (Hoelzel and Green 1992). Purification of PCR products was performed with a GFX™ PCR DNA and Gel Band Purification Kit (Amersham Pharmacia Biotech, Germany), following the manufacturer's instructions. Direct sequencing of both fragment strands was conducted through the use of a fluorescent dye-terminator cycle sequencing reaction (Thermo Sequenase™ Dye Terminator Cycle Sequencing Kit), using an ABI 377 Perkin Elmer automatic sequencer (Perkin Elmer Inc., USA). Twenty-two *X. kroyeri* samples from Atlantic populations and four from the Pacific coast of Panama were sequenced (Table 2).

## PCR/RFLP analysis of partial COI gene

We used the PCR/RFLP diagnostic system of Brazilian commercial shrimp species (Gusmão and Solé-Cava 2002) to examine 77 individuals previously identified as *Xiphopenaeus* 1 and 2 using allozymes. This was done to verify if there was a correspondence between the two *COI* PCR/RFLP haplotypes previously described for *Xiphopenaeus* Brazilian populations and the species detected by allozyme analyses. Twenty-six further PCR/RFLP haplotypes, from individuals that had not been analysed by allozymes, were also scored to verify the presence of polymorphisms within the endonuclease cleavage sites, and test the reliability of species identifi-

**Table 1** Enzyme and buffer systems analysed

| Enzyme | EC | Abbreviation | Buffer* |
|---|---|---|---|
| Alcohol dehydrogenase | 1.1.1.1 | ADH | TC7 |
| Adenylate kinase | 2.7.4.3 | AK | TC8 |
| Isocitrate dehydrogenase | 1.1.1.42 | IDH | TC7 |
| Lactate dehydrogenase | 1.1.1.27 | LDH | TEM |
| Malic enzyme | 1.1.1.40 | ME | TEM |
| Malate dehydrogenase | 1.1.1.37 | MDH | TC8 |
| Mannose 6-phosphate isomerase | 5.3.1.8 | MPI | TEM |
| Phosphogluconate dehydrogenase | 1.1.1.44 | PGD | TEM |
| Phosphoglucose isomerase | 5.3.1.9 | PGI | TC7 |
| Phosphoglucomutase | 5.4.2.2 | PGM | TC7 |
| Pro-Phe dipeptidase | 3.4.13.18 | PEP-A | TC8 |
| Tripeptidase (Leu-Gly-Gly) | 3.4.11.4 | PEP-B | TC8 |

*TEM = 0.10 M Tris, 0.01 M EDTA, 0.10 M maleate, pH 7.4 (Brewer 1970)
TC8 = 0.25 M Tris, 0.06 M citrate, pH 8.0 (Ward and Beardmore 1977)
TC7 = 0.135 M Tris, 0.043 M citrate, pH 7.0 (Shaw and Prasad 1970)

**Table 2** *Xiphopenaeus* spp. Correlation between *Xiphopenaeus* species 1 and 2 detected using allozymes and the PCR/RFLP haplotypes described by Gusmão and Solé-Cava (2002)

| Population | *Hinf* I/*Hinc* II haplotypes | | |
|---|---|---|---|
| | A/D *Xiphopenaeus* sp. 1 | E/C *Xiphopenaeus* sp. 2 | F/A *X. riveti* |
| Caracas (Venezuela) | 10* (3) | – | – |
| Natal (RN; Brazil) | 1 (1) | 14 (3) | – |
| Poças (BA; Brazil) | 15 (4) | – | – |
| Caravelas (BA; Brazil) | 12* (3) | – | – |
| Nova Almeida (ES; Brazil) | 15 (2) | – | – |
| Arraial do Cabo (RJ; Brazil) | 15 (2) | – | – |
| Ubatuba (SP; Brazil) | 6 (2) | 11 (2) | – |
| Panama City (Panama) | – | – | 4* (4) |
| Total | 74 (17) | 25 (5) | 4 (4) |

*N*–Number of individuals analysed per locality per species and corresponding *Hinf* I/*Hinc* II haplotypes; (*N*) Number of sequenced individuals per locality
*Individuals that were not analysed by allozymes

cation using the PCR/RFLP system. These included 12 individuals from Caravelas (Bahia), four from Panama City (Pacific Ocean) and ten from Caracas (Venezuela).

### COI data analyses

*COI* sequences were aligned using the Clustal X multiple alignment program Version 1.83 (Thompson et al. 1997) and alignments were confirmed by visual inspection and by translating the aligned DNA sequences. The species *Metapenaeus affinis* and *M. ensis* (Penaeidae family) (Quan et al. 2004; Accession Number AY264886; Lavery et al. 2004; AF279830) were used as the outgroups (Decapoda: Dendrobranchiata: Penaeoidea).

One additional *X. kroyeri* sequence from GenBank was also included in the analysis (GenBank Accession Number AY135200; R. Maggioni et al., Unpublished data). The geographical origin of the *X. kroyeri* sequence deposited by Maggioni et al. is not explicit on the database, but since that author has previously used *X. kroyeri* from the Atlantic (from Guaratuba in the South of Brazil and São Luis in the Northeast; R. Maggioni et al., Unpublished data) as the outgroup in a phylogenetic work using 16S sequences, we suspect that the GenBank *COI* sequence AY135200 has the same origin.

Basic statistics and phylogenetic analyses were conducted using the MEGA program Version 2.1 (Kumar et al. 2001) and PAUP 4.0 (Swofford 1998). Two tree building methods, maximum-likelihood (Felsenstein 1981) and neighbor-joining (Saitou and Nei 1987) were employed. For neighbor-joining analysis, sequence divergences between pairs of species were calculated using Kimura 2-parameter distance (Kimura 1980). The Modeltest program Version 3.06 (Posada and Crandall 1998) was used to evaluate the most appropriate model of DNA substitution for maximum-likelihood analyses of the data set. The best-fit model chosen after comparisons between likelihood scores from different DNA substitution models was the general time reversible with gamma distribution (GTR + G). This model incorporates unequal base frequencies (A = 0.2724; C = 0.2133; G = 0.1697; T = 0.3446), unequal substitution rates (A-C = 1.3337; A-G = 16.8523; A-T = 6.5833; C-G = 1.0892; C-T = 40.0940; G-T = 1.0000), and gamma distribution shape parameter of 0.3011. Starting tree(s) were obtained via neighbor-joining and a heuristic search was employed using the branch-swapping algorithm tree-bisection-reconnection (TBR). Branch support was assessed by bootstrapping the original data set using 1,000 replicates.

Two different statistical methods, Tajima's *D* statistic (Tajima 1989) and the McDonald-Kreitman (MK) test (McDonald and Kreitman 1991), implemented in the DNASP 4.0 computer program (Rozas et al. 2003) were used for testing neutrality of mutations. Tajima's *D* statistic tests neutrality based on the rationale that nucleotide polymorphism ($\theta$) and nucleotide diversity ($\pi$) values should be nearly equal under a neutral model of evolution, in which case *D* approaches zero. *D* values significantly different from zero suggest either balancing or diversifying selection, if positive, and purifying selection or recent population contraction, when negative. The MK test examines whether the ratio of both synonymous and non-synonymous sites are equivalent within and among species, by means of a 2×2 test of independence. Under neutrality, the ratios of polymorphic and fixed non-synonymous and synonymous substitution are expected to remain the same.

## Results

### Allozymes

One hundred and seventy four individuals of the five locations in Brazil were typed for 15 allozyme *loci* (Table 3). Strong heterozygote deficiencies ($F_{IS} = 0.575$; $P < 0.05$) were detected in the populations of Ubatuba (in

**Table 3** *Xiphopenaeus* spp. Allozyme allele frequencies and sample sizes (*N*).

| Locus | *Xiphopenaeus* sp. 1 | | | | | *Xiphopenaeus* sp. 2 | |
|---|---|---|---|---|---|---|---|
| | Natal | Poças | Nova Almeida | Arraial | Ubatuba | Natal | Ubatuba |
| *Ldh-2* | | | | | | | |
| (*N*) | (1) | (25) | (50) | (40) | (7) | (8) | (28) |
| A | – | – | – | – | – | 1.000 | 1.000 |
| B | 1.000 | 1.000 | 1.000 | 1.000 | 1.000 | – | – |
| *Mdh-1* | | | | | | | |
| (*N*) | (1) | (30) | (50) | (40) | (7) | (14) | (32) |
| A | 0.500 | 0.017 | 0.010 | 0.012 | – | – | – |
| B | 0.500 | 0.933 | 0.960 | 0.950 | 1.000 | 1.000 | 1.000 |
| C | – | 0.050 | 0.030 | 0.038 | – | – | – |
| *Mdh-2* | | | | | | | |
| (*N*) | (1) | (19) | (42) | (40) | (7) | (14) | (31) |
| A | – | 0.079 | 0.095 | 0.038 | 0.071 | – | 0.081 |
| B | 1.000 | 0.921 | 0.881 | 0.962 | 0.929 | 1.000 | 0.919 |
| C | – | – | 0.024 | – | – | – | – |
| *Me* | | | | | | | |
| (*N*) | (1) | (29) | (50) | (40) | (7) | (14) | (32) |
| A | – | – | – | – | – | – | 0.266 |
| B | – | – | 0.030 | – | – | 1.000 | 0.703 |
| C | 1.000 | 0.966 | 0.970 | 1.000 | 1.000 | – | 0.031 |
| D | – | 0.034 | – | – | – | – | – |
| *Mpi* | | | | | | | |
| (*N*) | (1) | (23) | (44) | (40) | (5) | (8) | (24) |
| A | – | – | – | – | – | 0.187 | 0.167 |
| B | – | 0.413 | 0.307 | 0.338 | – | 0.813 | 0.833 |
| C | 1.000 | 0.413 | 0.659 | 0.475 | 1.000 | – | – |
| D | – | 0.109 | 0.011 | 0.162 | – | – | – |
| E | – | 0.065 | 0.023 | 0.025 | – | – | – |
| *Pep-A* | | | | | | | |
| (*N*) | (1) | (30) | (50) | (40) | (6) | (14) | (32) |
| A | – | – | 0.030 | 0.025 | – | 0.071 | – |
| B | – | – | – | 0.013 | – | 0.893 | 0.984 |
| C | 1.000 | 1.000 | 0.970 | 0.962 | 1.000 | 0.036 | 0.016 |
| *Pep-B* | | | | | | | |
| (*N*) | (1) | (30) | (50) | (40) | (7) | (14) | (32) |
| A | 1.000 | 1.000 | 0.980 | 0.988 | 1.000 | – | – |
| B | – | – | 0.020 | 0.012 | – | 0.964 | 1.000 |
| C | – | – | – | – | – | 0.036 | – |
| *Pgd* | | | | | | | |
| (*N*) | (1) | (26) | (45) | (40) | (7) | (14) | (32) |
| A | 1.000 | 0.615 | 0.611 | 0.500 | 0.500 | – | 0.031 |
| B | – | 0.385 | 0.389 | 0.500 | 0.500 | 1.000 | 0.969 |
| *Pgi* | | | | | | | |
| (*N*) | (1) | (30) | (50) | (40) | (7) | (14) | (32) |
| A | – | – | 0.010 | 0.012 | – | 0.107 | – |
| B | – | 0.133 | 0.010 | 0.013 | – | 0.821 | 0.953 |
| C | 1.000 | 0.833 | 0.970 | 0.975 | 1.000 | 0.036 | 0.047 |
| D | – | 0.034 | 0.010 | – | – | 0.036 | – |
| *Pgm-1* | | | | | | | |
| (*N*) | (1) | (30) | (50) | (40) | (7) | (14) | (30) |
| A | – | – | 0.020 | – | – | – | 0.017 |
| B | – | – | – | – | – | 0.179 | 0.150 |
| C | 1.000 | 1.000 | 0.980 | 1.000 | 1.000 | – | – |
| D | – | – | – | – | – | 0.821 | 0.833 |

**Table 3** (Contd.)

| Locus | *Xiphopenaeus* sp. 1 | | | | | *Xiphopenaeus* sp. 2 | |
|---|---|---|---|---|---|---|---|
| | Natal | Poças | Nova Almeida | Arraial | Ubatuba | Natal | Ubatuba |
| *Pgm-2* | | | | | | | |
| (*N*) | (1) | (30) | (50) | (40) | (7) | (14) | (30) |
| A | 1.000 | 1.000 | 1.000 | 1.000 | 1.000 | – | – |
| B | – | – | – | – | – | 0.893 | 0.983 |
| C | – | – | – | – | – | 0.107 | 0.017 |
| $H_o$ | 0.067 | 0.114 | 0.105 | 0.092 | 0.019 | 0.101 | 0.105 |
| $H_e$ | 0.067 | 0.118 | 0.101 | 0.098 | 0.045 | 0.095 | 0.092 |

$H_o$ and $H_e$ observed and expected heterozygosities, respectively
The *Adh*, *Ak-1*, *Idh* and *Ldh-1* loci were monomorphic and identical in all populations analysed

the State of São Paulo) and Natal (in the State of Rio Grande do Norte). Those deficiencies were due to the presence of five loci (*Ldh-2*, *Mpi*, *Pep-2*, *Pgm-1*, *Pgm-2*; Table 3) that were fixed for alternative alleles in the same individuals within each population, indicating the existence of two different species in both regions. Multivariate analysis (FCA) of allelic data also yielded two very distinct groups of individuals, from the Ubatuba and Natal populations (first axis explains 42% of the variation, Fig. 2). One, called hereforth *Xiphopenaeus* sp. 1, was observed in each of the regions, ranging from Natal, where only one specimen was observed out of 15 samples analysed, to Ubatuba, where seven specimens were detected in a total of 39. Only individuals of this type were observed from Poças (in the State of Bahia) to Arraial do Cabo (in the State of Rio de Janeiro). Individuals of the second type, hereforth called *Xiphopenaeus* sp. 2, were abundant in Natal and Ubatuba, and were not found in any of the other locations. After demonstration of the presence of two cryptic Atlantic species, data analysis was done for each putative species separately, which resulted in no within-population deviations from Hardy-Weinberg expectations (*Xiphopenaeus* sp. 1: $F_{IS} = 0.029$, $P > 0.05$; *Xiphopenaeus* sp. 2: $F_{IS} = -0.124$, $P > 0.05$). Heterozygosity levels ($H_e = 0.02$–$0.11$; Table 3) were also similar to those observed in other penaeid species ($H_e = 0.006$–$0.130$; Mulley and Latter 1980; Lester 1983; Sunden and Davis 1991; Gusmão et al. 2000).

Population analysis of the most widely distributed species, *Xiphopenaeus* sp. 1 (with the exclusion of the Natal population due to a small sample size), resulted in significant $F_{ST}$ values ($F_{ST} = 0.020$–$0.122$; $P < 0.05$). This indicates that populations of this species are genetically structured along the studied area. Significant statistical differences ($F_{ST}$ and contingency table analyses; Table 4) were observed between the population from Nova Almeida (State of Espírito Santo) and those from Cabo Frio (RJ) and Poças (BA). Significant $F_{ST}$ values were also obtained in pairwise comparisons between the population from Ubatuba (SP), Cabo Frio (RJ), and Poças (BA). The populations of *Xiphopenaeus* sp. 2 from Natal and Ubatuba were also significantly different ($F_{ST} = 0.055$; $P < 0.01$).

Genetic identities (Nei 1978) between populations of the two cryptic Atlantic *Xiphopenaeus* species varied from 0.397 to 0.504, whereas within-species identity values were much higher: 0.953–0.999 for *Xiphopenaeus* sp. 1; and 0.994 for *Xiphopenaeus* sp. 2 (Table 5). The allozyme-based UPGMA similarity tree clearly shows the separation of the Brazilian samples in two cryptic Atlantic species (Fig. 3).

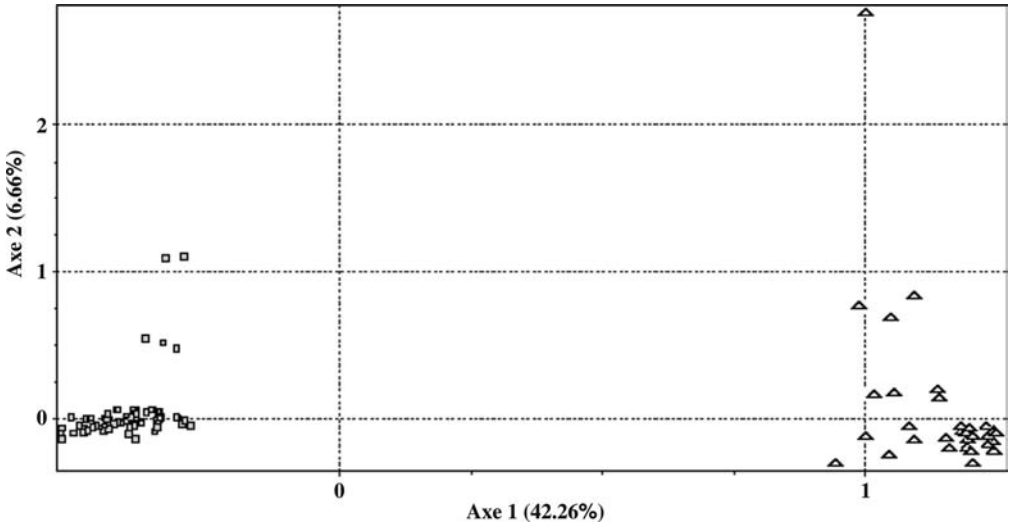


**Fig. 2** *Xiphopenaeus* spp. Multivariate (FCA) analysis of Atlantic *X. kroyeri* (after Pérez Farfante and Kensley 1997) populations based on allelic data from 15 allozyme loci

**Table 4** *Xiphopenaeus* sp. 1. Pairwise $F_{ST}$ values (above diagonal) and $\chi^2$ (contingency table; below diagonal) between populations

| Population | 1 | 2 | 3 | 4 |
|---|---|---|---|---|
| 1. Poças–BA | – | 0.024* | $0.010^{NS}$ | 0.122* |
| 2. N. Almeida–ES | 35.67** | – | 0.020* | $0.041^{NS}$ |
| 3. Arraial–RJ | $23.08^{NS}$ | 26.92* | – | 0.093* |
| 4. Ubatuba–SP | $16.13^{NS}$ | $8.35^{NS}$ | $11.91^{NS}$ | – |

$^{NS}$Not significant; $P > 0.05$
*$P < 0.05$
**$P < 0.01$

## PCR/RFLP analysis of partial COI gene

We found a complete correlation between the PCR/RFLP (*Hinf* I/*Hinc* II) *Xiphopenaeus* haplotypes (A/D and E/C; Gusmão and Solé-Cava 2002) and the two Brazilian sympatric species detected using allozymes (Table 2). This means that the previously developed molecular PCR/RFLP system can also be extended for the identification of the two Atlantic *Xiphopenaeus* species revealed in this study. PCR/RFLP analyses of the four Pacific individuals (*X. riveti*) revealed a third haplotype within *Xiphopenaeus* that is also different from all *COI* PCR/RFLP patterns described for Brazilian commercial shrimp species.

## COI sequence analysis

*Xiphopenaeus* spp. partial *COI* sequences (Fig. 4) were deposited in GenBank under Accession Numbers DQ084367–DQ084380. Sequence analyses show the existence of two Atlantic *Xiphopenaeus* clades, corresponding to the individual specimens analysed using allozymes, identified as *Xiphopenaeus* sp. 1 and *Xiphopenaeus* sp. 2. In addition, individuals from the Pacific Ocean formed a third distinct clade, showing that *X. riveti* is a valid species (see below). No gaps or stop codons were observed in any of the sequences. Within each putative species, the total numbers of segregating sites were eight, two and four for *Xiphopenaeus* sp. 1, *Xiphopenaeus* sp. 2 and *X. riveti* respectively, out of 621 sites analysed (207 codons). All intraspecific changes were synonymous substitutions. One fixed non-synonymous difference was observed between *X. riveti* and both *Xiphopenaeus* sp. 1 and *Xiphopenaeus* sp. 2, and two non-synonymous substitutions between *Xiphopenaeus* sp. 1 and *Xiphopenaeus* sp. 2. Seventy-two synonymous mutations were found between *Xiphopenaeus* sp. 1 and *Xiphopenaeus* sp. 2, 74 between *Xiphopenaeus* sp. 2 and *X. riveti*, and 59 between *Xiphopenaeus* sp. 2 and *X. riveti*. Fourteen different haplotypes (A-N; Fig. 5) were observed: seven within *Xiphopenaeus* sp. 1; three within *Xiphopenaeus* sp. 2; and four within *X. riveti*. The GC contents were 39.4, 36.2 and 37.3% for *Xiphopenaeus* sp. 1, *Xiphopenaeus* sp. 2 and *X. riveti* respectively. Neutrality tests failed to reject the null hypothesis that *COI* sequences were evolving in a neutral manner in the studied species (Tajima *D*: $P > 0.10$; MK Exact test: $P > 0.05$).

Pairwise levels of base divergence (Kimura 2-parameter distance) within each putative species were similar to those previously described for other penaeid species (less than 3%; Baldwin et al. 1998; Lavery et al. 2004; Quan et al. 2004), ranging from 0 to 0.008 within *Xiphopenaeus* sp. 1, from 0 to 0.003 within *Xiphopenaeus* sp. 2, and from 0.002 to 0.005 within *X. riveti*. Contrastingly, pairwise distances among the three *Xiphopenaeus* putative species varied from 0.106 to 0.114 between *Xiphopenaeus* sp. 2 and the Pacific *X. riveti*, from 0.136 to 0.151 between *X. riveti* and *Xiphopenaeus* sp. 1, and from 0.140 to 0.147 between the two Atlantic *Xiphopenaeus* species.

The tree topologies produced by both neighbor-joining and maximum-likelihood methods were congruent, showing the existence of three clearly different clusters with high support bootstrap values, corresponding to the two Atlantic (as seen in the allozyme analysis) and one Pacific *Xiphopenaeus* cryptic species (Fig. 4). The additional *X. kroyeri COI* sequence from the GenBank included in the study was identical to the most frequent haplotype of the most extensively distributed species, *Xiphopenaeus* sp. 1. The phylogenetic analyses indicate a sister-taxon relationship between *Xiphopenaeus* sp. 2 and *X. riveti*, but further sampling in the Atlantic and especially in the Pacific is necessary to investigate the existence of other cryptic species that may eventually modify the present tree topology.

# Discussion

The detection of two highly diverged *COI* clades (Fig. 4) combined with the presence of five allozyme diagnostic

**Table 5** *Xiphopenaeus* spp. Pairwise values of unbiased genetic identities (*I* above diagonal) and distances (*D* below diagonal) (Nei 1978) between populations

| Population | 1 | 2 | 3 | 4 | 5 | 6 | 7 |
|---|---|---|---|---|---|---|---|
| 1. *Xiphopenaeus* sp. 2 (Natal–RN) | | 0.397 | 0.504 | 0.501 | 0.488 | 0.466 | 0.994 |
| 2. *Xiphopenaeus* sp. 1 (Natal–RN) | 0.924 | | 0.955 | 0.953 | 0.966 | 0.967 | 0.395 |
| 3. *Xiphopenaeus* sp. 1 (Poças–BA) | 0.685 | 0.046 | | 0.999 | 0.997 | 0.982 | 0.502 |
| 4. *Xiphopenaeus* sp. 1 (Arraial–RJ) | 0.691 | 0.049 | 0.001 | | 0.998 | 0.987 | 0.497 |
| 5. *Xiphopenaeus* sp. 1 (N. Almeida–ES) | 0.717 | 0.035 | 0.003 | 0.002 | | 0.994 | 0.485 |
| 6. *Xiphopenaeus* sp. 1 (Ubatuba–SP) | 0.763 | 0.034 | 0.019 | 0.013 | 0.006 | | 0.462 |
| 7. *Xiphopenaeus* sp. 2 (Ubatuba–SP) | 0.006 | 0.930 | 0.690 | 0.699 | 0.724 | 0.772 | |

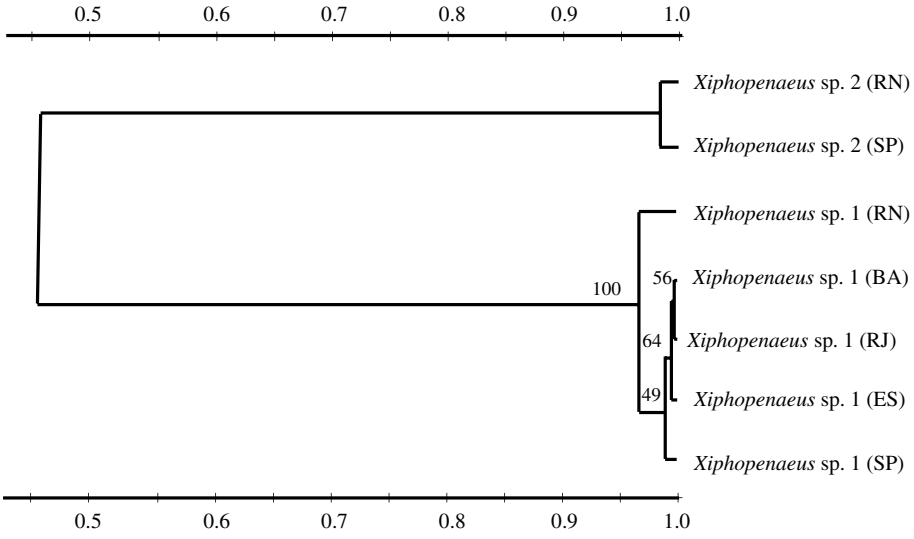


**Fig. 3** *Xiphopenaeus* spp. Allozyme-based UPGMA similarity *tree* showing genetic relatedness (gene identity, *I*) of Atlantic samples. *Numbers* above branches represent bootstrap values based on 1,000 replications

loci in sympatric populations of the seabob shrimp clearly show that *X. kroyeri* from the West Atlantic comprises two different species. Furthermore, *COI* sequence data confirm that the Pacific *X. riveti* is a valid species and, thus, should not be synonymized with *X. kroyeri* as suggested earlier (Pérez Farfante and Kensley 1997). Each Atlantic *Xiphopenaeus* species corresponds to one of the two PCR/RFLP haplotypes previously observed during the development of a molecular diagnostic system for identification of shrimp commercial products (Gusmão and Solé-Cava 2002). Individuals from the Pacific Ocean presented a third haplotype, never observed among the Atlantic specimens (Table 2).

The two Atlantic species here referred to as *Xiphopenaeus* sp. 1 and *Xiphopenaeus* sp. 2, had fixed nucleotide differences in mitochondrial *COI* sequences and five nuclear diagnostic allozyme loci. The *Xiphopenaeus* individuals from the Pacific Ocean (*X. riveti*, considered a synonym of *X. kroyeri* by Pérez Farfante and Kensley 1997) also presented fixed *COI* differences when compared to *Xiphopenaeus* sp. 2 and *Xiphopenaeus* sp. 1.

The intraspecific variation at the *COI* gene (less than 1%) was similar to sequence divergences found within other penaeid species, but the levels of congeneric sequence divergence found among *Xiphopenaeus* cryptic species, ranging from 11 to 15%, are unexpectedly high, comparable to those observed between phylogenetically close Atlantic shrimp genera (13–15% between *Farfantepenaeus* and *Litopenaeus*; Gusmão et al. 2000). Indication of cryptic speciation within penaeids was also revealed via *COI* sequence analysis of two morphologically indistinguishable clades within *Fenneropenaeus merguiensis* that had average sequence divergences of 5% (Hualkasin et al. 2003).

Large discrepancies between morphological and molecular divergences have been also documented between other morphologically similar (but not cryptic) penaeid species, indicating possible accelerated mitochondrial evolution or morphological stasis. This might have been caused by stabilizing selection on morphological or physiological features (Palumbi and Benzie 1991).

The rate of *COI* gene evolution has been estimated as approximately 3% sequence divergence per million years (MY) for *Penaeus* species based on the comparison of sister taxa across the Isthmus of Panama (Baldwin et al. 1998). Using this rate, *Xiphopenaeus* sp. 1 may have diverged from the ancestor of *Xiphopenaeus* sp. 2 and *X. riveti* at around 4.5 MY while the *Xiphopenaeus* sp. 2 and the Pacific species *X. riveti* may have been evolving independently from each other since about 4 MY ago. Similar rates of *COI* evolution, of 1.2–3.7% sequence divergence per MY, can also be deduced from average pairwise K2P divergences between the transisthmian sister species *Xiphopenaeus* sp. 2 and *X. riveti*, considering that the uplift of the Isthmus of Panama effectively isolated the faunas of the Atlantic and Pacific Oceans between 3 and 9 million years ago (Baldwin et al. 1998; Knowlton and Weigt 1998; Marko 2002). Thus, *X. riveti* may have originated from an Atlantic ancestor as a consequence of the rising of the Isthmus, during the Pliocene.

The two Atlantic species seem to have different distributions and abundances along the studied area, but further studies are needed to elucidate the actual distribution of *Xiphopenaeus* sp. 2. *Xiphopenaeus* sp. 1 was observed in all sampling sites ranging from Ubatuba (São Paulo State) to Caracas (Venezuela), and probably has a continuous distribution along the coast. The type locality of *X. kroyeri* is in Rio de Janeiro (RJ; Heller 1862), where only *Xiphopenaeus* sp. 1 was found. It is likely, thus, that *Xiphopenaeus* sp. 1 is *X. kroyeri*. *Xiphopenaeus* sp. 2, on the other hand, was only observed (although in greater number than sp. 1) in the Northern and Southernmost Brazilian sampling sites of Natal and Ubatuba. This can be an indication of a marked discontinuity in the distribution of that species, but its absence in the other regions can also be explained by (1) seasonal variation on the composition of species

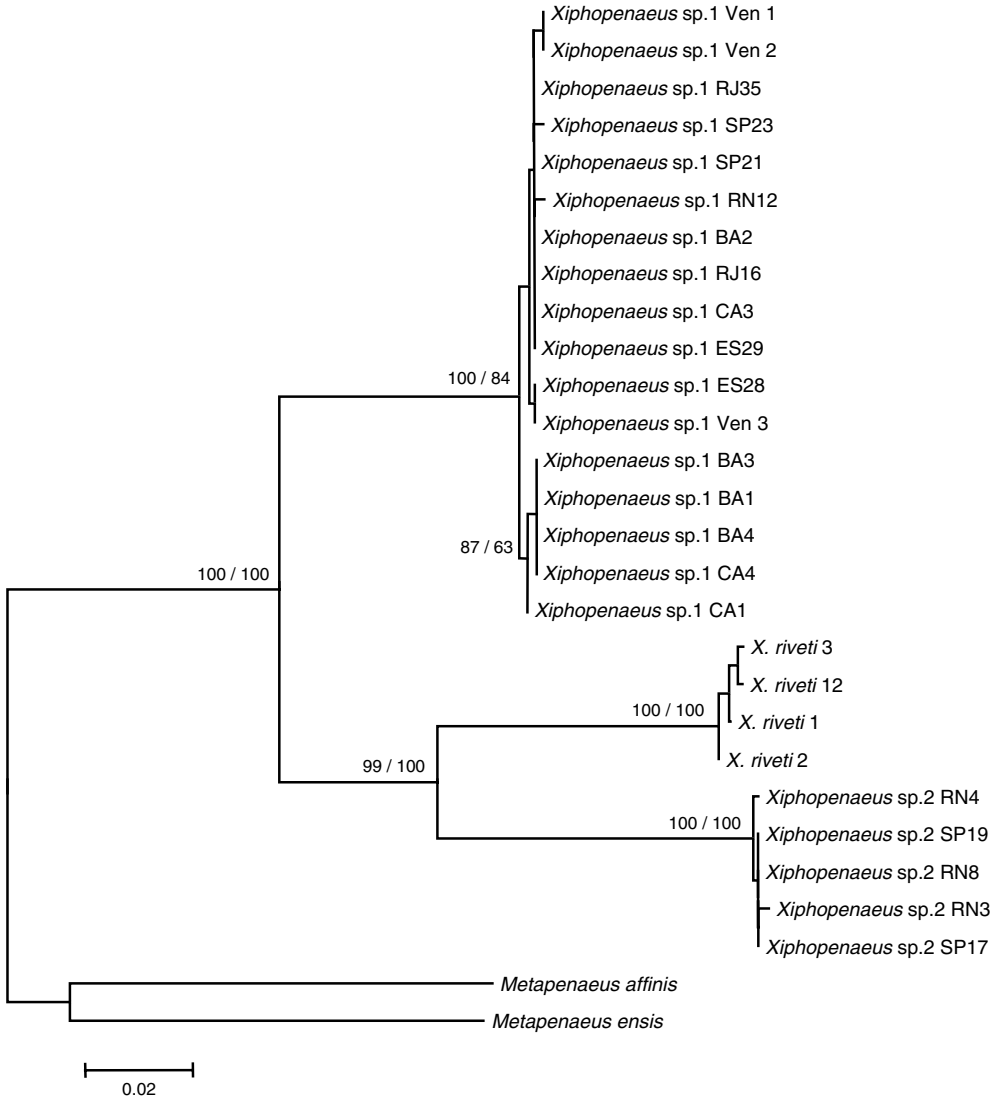


**Fig. 4** *Xiphopenaeus* spp. Cytochrome oxidase I-based neighbor-joining tree and maximum likelihood tree. Numbers above branches are bootstrap values (1,000 replicates) for neighbor-joining and maximum likelihood trees, respectively

among regions (as only one sampling was made in each location), (2) differences in abundance of these resources among areas due to ecological constraints or (3) stock size reduction caused by overexploitation, or (4) due to a combination of the previous factors. Seasonal abundance variation was previously reported for *Xiphopenaeus* populations from Ubatuba Bay (Nakagaki and Negreiros-Fransozo 1998) that presented low numbers during summer (December–March), probably due to the incoming of the South Atlantic central water (cold current $< 18°C$) during this period. Studies on the biology of the genus also show that it prefers to inhabit the coastline or estuaries with mud or mud/sand sediments, which are close to the coast and, which are under the influence of permanent river discharges (Paiva et al. 1971 but see Castro et al. 2005). Although seasonal and ecological constraints were already reported, stock reduction due to fishery activities may also explain the discontinuous distribution of *Xiphopenaeus* sp. 2. Since the two Atlantic *Xiphopenaeus* species were, until now, regarded as a single entity, their exploitation and fishery statistics have been done indiscriminately. Annual production data on "*X. kroyeri*" between 1964 and 1994 show a significant reduction in relative abundance between 1990 and 1991, with worrying signs of overexploitation in the South and Southeast regions of Brazil (Neto and Dornelles 1996), and recent data on production indicate that *Xiphopenaeus* fisheries are above sustainable limits. Fishery activities beyond sustainable limits coupled with presence of unknown cryptic species can lead to the disappearance of resources in regions where the fishing activities are more intense or the resource is less abundant (Thorpe et al. 2000). If *Xiphopenaeus* sp. 2 is more sensitive to exploitation than *Xiphopenaeus* sp. 1, it may have already been wiped out from many areas.

One of the implications of this work is that fishery biologists will need to take into account that they are dealing with two different cryptic seabob shrimp species in the Southwest Atlantic. Although no morphological

| | 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 2 2 2 |
|---|---|
| | 1 1 1 3 4 5 5 6 6 6 7 8 8 9 0 0 0 1 1 2 3 4 5 5 5 6 6 7 7 8 8 9 9 0 1 2 |
| | 6 9 2 5 8 0 5 1 4 3 6 9 2 4 7 0 2 5 8 1 2 0 5 7 0 3 9 2 8 1 7 3 9 2 8 1 3 5 |
| *Xiphopenaeus* sp.1 (A-G) | C G C C C C G C T C A C Y A A T C T A C C G T C T A A G T C A T A A C C C T |
| *Xiphopenaeus* sp.2 (H-J) | T A T . T . M T . T G T T G G . T C . T T . C T A G . C . T . . T G . T T A |
| *Xiphopenaeus riveti* (K-N) | T A . T . A T T C T . . . G G C T C G T T A . T A . G A Y T G C . . T T . A |

| | 2 2 2 2 2 2 2 2 2 2 2 2 2 3 3 3 3 3 3 3 3 3 3 3 3 3 3 3 3 3 3 3 3 4 4 4 |
|---|---|
| | 3 4 5 6 6 7 7 7 8 8 9 9 9 0 0 0 1 1 2 2 2 3 3 4 4 4 5 7 7 8 8 8 9 9 9 0 0 0 |
| | 4 9 2 1 4 1 3 6 2 5 1 4 5 0 3 9 5 8 1 4 7 0 9 0 5 8 8 5 8 1 4 7 3 6 9 0 5 8 |
| *Xiphopenaeus* sp.1 (A-G) | C A T T C C A T Y C C A C C Y T C R G C Y A C C T A C T T C T A C T C C C C |
| *Xiphopenaeus* sp.2 (H-J) | T G C . T . G C . T . G T T . C T . A T T G T T . T . . A T Y . . . T T . T |
| *Xiphopenaeus riveti* (K-N) | T . . C T T . C . T T G T T . . T . A T . G . T C T T C A T C G T Y T T T . |

| | 4 4 4 4 4 4 4 4 4 4 4 5 5 5 5 5 5 5 5 5 5 5 5 5 5 5 5 5 5 5 5 6 6 6 |
|---|---|
| | 1 2 2 4 4 5 6 7 7 7 9 0 0 1 1 1 3 4 4 4 5 5 6 6 7 7 8 8 8 9 9 9 9 0 0 1 |
| | 7 0 6 1 7 9 8 1 4 8 2 1 7 0 6 9 7 0 3 6 5 8 7 8 7 9 2 5 8 1 4 7 8 6 9 5 |
| *Xiphopenaeus* sp.1 (A-G) | C A T C R A T C C A T T C T C C C C C T T A C A T T C A T C R Y T A G C |
| *Xiphopenaeus* sp.2 (H-J) | T G . T A C C T . G C C T . T T T T . C C . T G . C T . C T A . . T A T |
| *Xiphopenaeus riveti* (K-N) | . . C . C T C . T G . . . C R . . . T . C G T . Y C T G C T A . C T A T |

**Fig. 5** *Xiphopenaeus* spp. Polymorphic nucleotide sites of fourteen *COI* haplotypes (A-N) of the three *Xiphopenaeus* species. (.) Identical nucleotide; (R) A or G; (Y) C or T; (M) A or C

differences are available to distinguish the two species in the field, the geographical discontinuity of *Xiphopeaeus* sp. 2, which was only observed in Natal and Ubatuba together with *Xiphopenaeus* sp. 1, may be considered in future management of fisheries in these two regions. Furthermore, the populations of *Xiphopenaeus* sp. 1 from Nova Almeida (State of Espírito Santo) represent a separate stock of seabob shrimp from those from Cabo Frio (State of Rio de Janeiro) and Poças (State of Bahia) (Table 4). The *Xiphopenaeus* sp. 2 populations from Natal and Ubatuba also comprise two genetically different fishery stocks ($F_{ST} = 0.055$; $P < 0.01$).

The large genetic differences observed among the three *Xiphopenaeus COI* lineages, as well as the presence of five diagnostic allozyme loci between sympatric samples from Brazil, are at odds with the conclusion of Pérez Farfante and Kensley (1997) about the monotypy of this genus. The data show that the genus should include at least three species: *Xiphopenaeus* sp. 1 and *Xiphopenaeus* sp. 2 for the Atlantic, and *X. riveti* for the Pacific coast of the Americas. According to Bouvier (1907), *X. riveti* could be distinguished from *X. kroyeri* on the basis of the number of dorsal teeth: the rostrum of *X. riveti* is armed with four dorsal teeth and a fifth rudimentary anterior one, whereas *X. kroyeri* would have five fully formed dorsal teeth. However, we observed some variation on the number of teeth among Atlantic individuals: two of the seventeen specimens analysed, both collected at Poças—Bahia, were found to have only three and four well developed dorsal teeth. Nevertheless, both individuals grouped with the other *Xiphopenaeus* sp. 1 specimen in both allozyme and *COI* analyses, indicating that this feature is probably not adequate for morphological distinction between Atlantic and Pacific species.

Although nine genera from the Penaeidae family occur in Brazilian waters (as per Pérez Farfante and Kensley 1997), members of only three (commercial) genera (*Farfantepenaeus*, *Litopenaeus*, *Xiphopenaeus*) have hitherto been studied using molecular techniques. The analysis of the five Brazilian species within those genera (*F. brasiliensis*, *F. paulensis*, *F. subtilis*, *Litopenaeus schmitti*, and *X. kroyeri*) has already revealed that two of them (*F. subtilis* and *X. kroyeri*) concealed cryptic species (Gusmão et al. 2000; present work, respectively). This is an indication that the actual number of penaeid species has been underestimated either due to the lack of diagnostic morphological characters or to an over conservative taxonomy, as has already been reported for several marine invertebrates (Knowlton 2000; Thorpe et al. 2000). As with *Xiphopenaeus*, other genera comprising species with broad distributions (for instance, *Funchalia* and *Pelagopenaeus*) may need re-examination.

Given the detection of a new (and cryptic) *Xiphopenaeus* species within the Southwest Atlantic, it would be interesting to investigate the possibility that the Pacific *X. riveti*, which supposedly ranges from the coasts of Mexico to Peru, may also conceal cryptic taxa.

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