Marine Biology (2000) 137: 435–446

J. Gusmão · C. Lazoski · A. M. Solé-Cava

A new species of *Penaeus* (Crustacea: Penaeidae) revealed by allozyme and cytochrome oxidase I analyses

Received: 6 January 2000 / Accepted: 13 June 2000

Abstract Penaeid shrimps comprise an important portion of the world's industrial fisheries and mariculture. In the Southwest Atlantic, *Penaeus* (*Farfantepenaeus*) *subtilis*, *P.* (*F.*) *paulensis*, *P.* (*F.*) *brasiliensis* and *P.* (*Litopenaeus*) *schmitti* are the most important commercially exploited species. Despite their high commercial value, there is little information available on the different aspects of their biology or genetics and almost no data on stock structure. Also, the taxonomic status of *P. subtilis* populations in relation to *P. paulensis* and their geographic ranges have been recently questioned. In this paper we used both nuclear (allozymes) and mitochondrial (cytochrome oxidase subunit 1) data to compare species of south-western Atlantic penaeids and to estimate their phylogenetic relationships. We could readily discriminate among the four main Brazilian penaeid species and detected a fifth, new, species of *Penaeus*. This new species corresponded to one of the described morphotypes of *P. subtilis*. Based on these analyses, the geographic ranges of *P. subtilis* and *P. paulensis* were clarified. Our data also support the conclusion of Baldwin et al. (1998) that the subgenus *Farfantepenaeus* is polyphyletic.

Communicated by J. P. Thorpe, Port Erin

J. Gusmão · C. Lazoski · A. M. Solé-Cava (✉)
Laboratório de Biodiversidade Molecular,
Departamento de Genética, Instituto de Biologia,
UFRJ, Bloco A, CCS
Ilha do Fundão 21941-490, Rio de Janeiro, Brazil

Fax: 0055 21 562-6397
e-mail: sole@centroin.com.br

A. M. Solé-Cava
Department of Environmental and Evolutionary Biology,
University of Liverpool,
Port Erin Marine Laboratory, Isle of Man, UK

Introduction

Penaeid shrimps comprise an important portion of the world's fisheries and mariculture (Sunden and Davis 1991). In 1997, shrimps represented over 10% of all fish and shellfish export revenues in the world (FAO 1999). In the Southwest Atlantic, the main commercially fished penaeids were four species in the genus *Penaeus* Fabricius, 1798: *P. subtilis*, *P. paulensis*, *P. brasiliensis* and *P. schmitti*. Despite the high commercial value of these Brazilian penaeid species, there is little information available on the different aspects of their biology or genetics, and almost no data on stock structure.

According to Pérez Farfante (1969), *Penaeus subtilis* ranges from Rio de Janeiro north to the Caribbean, *P. paulensis* ranges from Buenos Aires (Argentina) to Rio de Janeiro (Brazil), and *P. brasiliensis* and *P. schmitti* can be found from southern Brazil to the Caribbean. However, these geographic ranges remain poorly defined, and the taxonomy of some of the commercially important species of *Penaeus* also has been recently questioned. In particular, *P. subtilis* was considered to exist in two morphologically distinguishable populations, one ranging from the Caribbean (as far North as Cuba) to Rio de Janeiro, and the other from Gulf of Paria (Venezuela; 10°18′N; 62°05′W) to off Camocim (Brazil; 02°54′S; 40°50′W, see Fig. 1) (Pérez Farfante 1967, 1969). According to Pérez Farfante (1967), shrimps in the latter population (here called "Morphotype I", or "MI"), have a shorter and much shallower adrostral sulcus, tapering more posteriorly, and a longer and distinctly sinuous rostrum. At the western and eastern ends of their distribution, specimens of MI with the above characteristics are intermingled with others having a longer and wider adrostral sulcus and a slightly shorter and less sinuous rostrum ("Morphotype II", MII). Pérez Farfante suggested that environmental conditions could be acting to produce the observed differences in morphology. However, a study using isoelectric focusing of sarcoplasmic proteins of

Fig. 1 *Penaeus* spp. Distribution and sampling sites (o) on eastern coast of South America

samples from that area (States of Ceará and Rio Grande do Norte) suggested that the two described populations (MI and MII) might belong to different species (Maggioni 1996). Those two morphotypes were subsequently studied through allozyme electrophoresis, which confirmed that they were probably not conspecific (D'Incao et al. 1998). Because of the high similarity found between the allozymes of Morphotype II of *P. subtilis* and those of *P. paulensis*, D'Incao et al. concluded that these were conspecific, thus extending by 2700 km north to Fortaleza (northeast of Brazil: Fig. 1), the apparent distribution of the latter species (D'Incao et al. 1998). D'Incao et al.'s study, however, was limited to just two localities, and did not consider possible geographic variation in gene frequencies within each species. Given the importance of both *P. subtilis* and *P. paulensis* for the shrimp fishing industry of Brazil, it is fundamental that their real geographic distributions be known, so that legislation for the control of their fisheries can be used effectively.

A recent phylogenetic and biogeographic study, using mitochondrial cytochrome oxidase subunit 1 (CO1) gene sequences, indicated that some of the subgenera of *Penaeus* are not monophyletic (Baldwin et al. 1998). In the CO1-based tree, four studied species of Western Atlantic *Penaeus*, currently assigned to two different worldwide subgenera, appear as a single monophyletic unit (Baldwin et al. 1998). This indicates that biogeography may be more important than morphology for the separation of monophyletic groups in the genus. However, Baldwin et al. cautioned that taxonomic changes should not be made "based on the sequence analysis of one single gene". Also, important Western Atlantic representatives of both putative subgenera, such as *P.* (*Farfantepenaeus*) *subtilis* and *P.* (*Litopenaeus*) *schmitti* were not included, thus leaving gaps in the data. Resolution of the subgeneric phylogeny is particularly important in light of the recent proposal that the subgenera of *Penaeus* should be raised to generic level (Pérez Farfante and Kensley 1997).

In this paper we used both nuclear (allozymes) and mitochondrial (cytochrome oxidase subunit 1) data to compare the different species of Southwest Atlantic commercially important penaeids, and to estimate their phylogenetic relationships. Using 1 mitochondrial and 14 nuclear genes, we could readily discriminate between the four main Brazilian penaeid species and detected a fifth, new, species of *Penaeus*. This new species corresponded to one of the morphotypes of *P. subtilis*, and was genetically different from any of the other species studied. Our data also support the conclusion of Baldwin et al. (1998) that the subgenus *Farfantepenaeus* is polyphyletic. Finally, we have used a restriction fragment-length polymorphism (RFLP) analysis of the CO1 gene to obtain species-specific markers, which will be useful for the identification of larvae and industrialised products of each species.

Materials and methods

Samples of *Penaeus subtilis*, *P. paulensis*, *P. brasiliensis* and *P. schmitti* were collected from eight different sites (Fig. 1) along >4000 km of the Brazilian coast, between October 1997 and September 1999. Samples were stored on dry ice and transported to the laboratory, where they were identified morphologically (after Pérez Farfante 1969). Individuals of both morphotypes of *P. subtilis* were identified based on adrostral sulcus characteristics, as indicated in Fig. 2. Muscle tissue was preserved at −20 °C

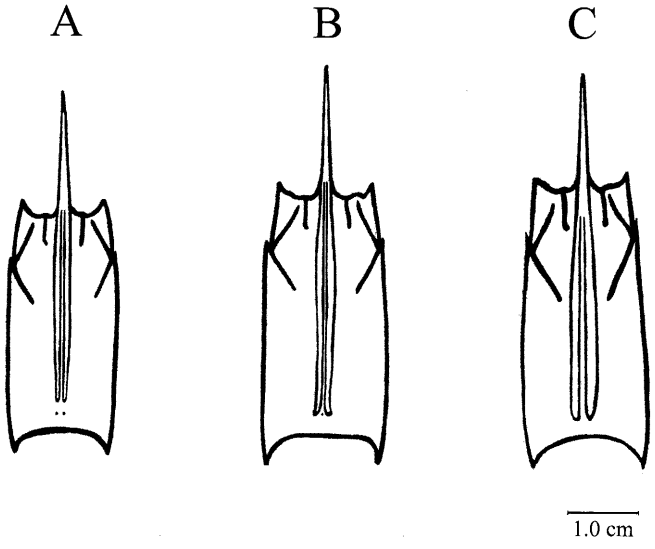

Fig. 2 *Penaeus* spp. Adrostral sulci characteristics of *P. subtilis* Morphotypes I and II (*A* and *B*) and *P. paulensis* (*C*)

in a solution containing 5% sodium dodecyl sulphate (SDS), 250 m*M* EDTA and 50 m*M* Tris HCl, pH 8.0, for DNA extraction, or in liquid nitrogen until required for allozyme analysis. Total DNA extraction was performed using a proteinase K, SDS, phenol-chloroform method (Garcia and Benzie 1995).

Allozyme analysis

Allozymes were analysed using 12.5% starch gel electrophoresis and standard methodology (Harris and Hopkinson 1978; Murphy et al. 1990). The 3 buffer systems and 11 enzyme systems investigated are summarised in Table 1. Allozyme patterns were visualised using standard enzyme stains (Shaw and Prasad 1970; Manchenko 1994). Genotype frequencies were used to estimate gene frequencies, heterozygosities, unbiased genetic identities and distances (*I* and *D*, respectively: Nei 1978), and UPGMA trees (Sneath and Sokal 1973) using the BIOSYS programme, Version 1.7 (Swofford and Selander 1981).

Sequencing of cytochrome oxidase 1

Sequencing methods followed standard procedures (Hoelzel and Green 1992). A section of the 3'end of the mitochondrial cytochrome oxidase subunit 1 gene was amplified using primers CO9 (6607) [5′-TTCGGTCA(T/C)CCAGAAGT(C/A)TAT] and CO10 (7214) [5′-TAAGCGTCTGGGTAGTCTGA(A/G)TA(T/G)CG] (Baldwin et al. 1998). The respective positions of the primers used in the homologous region of the human genome are indicated in parenthesies. Polymerase chain-reactions (PCR) used 10 ng of template DNA, 1 unit of Taq polymerase (Pharmacia), 200 µ*M* each of the four dinucleotides, 200 n*M* of each primer, in 25 µl of 1× PCR buffer (Pharmacia). Amplifications were performed in a Mini-cycler (MJ Research) programmed for one denaturation step at 94 °C for 3 min, followed by 35 cycles at 94 °C for 1 min, 45 °C for 1 min, and 72 °C for 1 min, and a final 5 min extension step at 72 °C. To detect possible contamination, negative controls, consisting of template-free reactions, were included in all PCR amplifications. PCR reactions produced one single, strong band of the expected size for most species studied. The one exception was *Penaeus schmitti*, for which three faint bands (820, 600 and 490 base pairs, bp) were consistently produced with the primers used. Sequencing of the 600 bp band revealed that this was probably the result of the amplification of a nuclear pseudogene, since it had a 90 to 99% similarity with Western Atlantic shrimp CO1 sequences, but included one insertion and stop-codons. *P. schmitti* was, consequently omitted from subsequent DNA analyses.

Purification of amplified products was performed on Qiagen spin columns (Qiagen) or using a GFX™ PCR DNA and Gel Band Purification Kit (Amersham Pharmacia Biotech), according to the manufacturer's instructions. Direct sequencing of both strands of the amplified DNA molecule was conducted through the use of a fluorescent dye-terminator cycle sequencing reaction (Thermo Sequenase™ Dye Terminator Cycle Sequencing Kit), based on the chain-termination dideoxynucleotide method of Sanger and coworkers (Sanger et al. 1977), using Perkin Elmer automatic sequencers ABI 310 and 377 (ABI Perkin Elmer).

Table 1 Enzyme and buffer systems analysed (*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); *TEM* 0.10 *M* Tris, 0.01 *M* EDTA, 0.10 *M* maleate, pH 7.4 (Brewer 1970))

Enzyme	E.C. No.	Abbrev.	Buffer
Adenilate 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	1.1.1.37	*Me*	TEM
Malic dehydrogenase	1.1.1.37	*Mdh*	TC8
Mannose 6-phosphate isomerase	5.3.1.8	*Mpi*	TEM
Peptidases (pro-phe)	3.4.1.1	*Pep*	TC8
Peptidases (leu-gly-gly)	3.4.1.1	*Pep*	TC8
Phosphogluconic dehydrogenase	1.1.1.44	*Pgd*	TEM
Phosphoglucose isomerase	5.3.1.9	*Pgi*	TC7
Phosphoglucomutase	5.4.2.2	*Pgm*	TC7

For phylogenetic analyses of sequence data, at least one individual of each composite haplotype found by PCR/RFLP analysis for each species was used. Sequence analyses included 5 *Penaeus subtilis* MI (4 from Fortaleza and 1 from Atins), and 4 *P. subtilis* MII (2 from Rio de Janeiro and 1 each from Recife and Fortaleza), 5 *P. paulensis* (4 from Rio de Janeiro and 1 from Lagoa dos Patos), 3 *Penaeus brasiliensis* specimens (from Recife, Ilhéus and Itajaí). These sequences were further compared with those available in GenBank (Accession Nos. AF029392, AF029393, X84355, AF014376–AF014383, AF014385, AF029390 and AF029391), which included the Western Atlantic species *P. paulensis*, *P. brasiliensis*, *P. notialis*, *P. duorarum* and *P. setiferus*; the Indo-Pacific *P. japonicus*, *P. canaliculatus*, *P. indicus*, *P. merguiensis*, *P. monodon* and *P. semisulcatus*; the Eastern Atlantic *P. kerathurus*; and the Eastern Pacific *P. vannamei* and *P. stylirostris*. *Sicyonia ingentis* (family Sicyoniidae) was used as an outgroup (Accession No. AF014384). Sequences of these species were aligned using the Clustal W programme, Version 1.5 (Thompson et al. 1994), and confirmed by visual inspection. Sequence-analyses were conducted to infer the phylogenetic relationships among species, using the neighbor-joining (NJ; Saitou and Nei 1987) algorithm, based on the pairwise proportion of nucleotide differences (*p* distances). Confidence probability values (CP) were estimated for the constructed NJ tree (Rzhetsky and Nei 1992). Distances between sequence-pairs were <0.2, so no corrections for TS:TV (transition:transversion) ratios were necessary (Kumar et al. 1994). However, Kimura two-parameter distances (Kimura 1980) were computed for the estimation of time since species separation, based on the clock calibration for the shrimp genus *Alpheus* (Knowlton and Weigt 1998). All calculations were performed using the MEGA programme, Version 1.02 (Kumar et al. 1994).

RFLP analysis of the CO1 gene

The DNA sequences obtained for each nominate species were initially analysed using the online Webcutter programme, Version 1.0 (written by M. Heiman, and available at http://www.medkem.gu.se/cutter/), to search for restriction enzyme sites producing patterns that might differentiate among the Brazilian species. Four enzymes – *AluI*, *NdeII*, *BglII* and *HhaI* – were thus selected to analyse different individuals of each species. In order to verify any possible intraspecific variation, 2 to 20 specimens were used for each species and sampling site (making a total of 178 individuals). The RFLP analyses of the amplified products followed standard procedures (Chow et al. 1993), using the unpurified products of the PCR reactions (5 µl of PCR reaction per 15 µl restriction reaction). Restriction reactions were conducted as recommended by the supplier (GibcoBRL®) for 4 h at 37 °C, and the digestion products were separated by electrophoresis for 3 h in 2% agarose gels with TBE buffer (Sambrook et al. 1989). The gels were then stained with ethidium bromide and photographed on an UV transilluminator using a Kodak DC - 40 digital system. Banding patterns were analysed by eye, and the restriction maps produced for the different haplotypes were confirmed using the sequence data. Haplotypic diversity (*h*) was calculated for each species by the method of Nei (1987).

Results

Allozymes

A total of 484 samples from eight localities were typed for allozymes coding for 14 loci (Table 2). Significant

Table 2 *Penaeus* spp. Allele frequencies and sample sizes (N) at 14 loci and 14 populations (H_o, H_e observed and expected heterozygosities, respectively)

Locus	*P. subtilis* MI		*P. subtilis* MII				*P. paulensis*			*P. brasiliensis*				*P. schmitti*
	Atins	Fortaleza	Fortaleza	Recife	Ilhéus	Rio	Rio	Santos	L. Patos	Fortaleza	Ilhéus	Rio	Itajaí	Recife
Ak														
(N)	(38)	(36)	(17)	(29)	(33)	(2)	(22)	(27)	(38)	(63)	(33)	(48)	(30)	(33)
A	0.20	0.24	0	0	0	0	0	0	0	0	0	0	0	1.00
B	0	0	0.03	0	0	0	0	0	0	0.02	0	0	0	0
C	0.80	0.76	0	0	0	0	0	0	0	0	0	0	0	0
D	0	0	0.97	1.00	1.00	1.00	1.00	1.00	1.00	0.98	1.00	1.00	1.00	0
Idh														
(N)	(38)	(36)	(15)	(30)	(49)	(2)	(22)	(27)	(40)	(77)	(33)	(48)	(30)	(35)
A	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
Ldh														
(N)	(38)	(37)	(17)	(30)	(49)	(2)	(22)	(27)	(40)	(80)	(33)	(48)	(30)	(35)
A	0.01	0	0	0	0	0	0	0	0	0	0	0	0	0
B	0.99	1.00	1.00	1.00	0.99	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
C	0	0	0	0	0.01	0	0	0	0	0	0	0	0	0
Mdh-1														
(N)	(40)	(37)	(17)	(30)	(49)	(2)	(22)	(27)	(40)	(80)	(33)	(48)	(30)	(35)
A	0	0	0	0	0	0	0	0	0.01	0	0.02	0	0	0
B	1.00	0	0	0	0	0	0	0	0	0	0	0	0	0
C	0	1.00	0.97	1.00	1.00	1.00	1.00	1.00	0.99	0.98	0.98	1.00	1.00	100
D	0	0	0.03	0	0	0	0	0	0	0.01	0	0	0	0
E	0	0	0	0	0	0	0	0	0	0.01	0	0	0	0
Mdh-2														
(N)	(40)	(37)	(17)	(30)	(49)	(2)	(22)	(27)	(40)	(80)	(33)	(48)	(30)	(35)
A	0	0	0	0	0	0	0	0	0	0	0	0	0	1.00
B	0	0.01	0.09	0	0.01	0	0	0	0	0	0	0	0	0
C	0	0.05	0	0.02	0	0	0	0.04	0	0.01	0	0	0	0
D	0.93	0.86	0.91	0.98	0.99	1.00	1.00	0.96	1.00	0.99	0.98	0.99	1.00	0
E	0.07	0.08	0	0	0	0	0	0	0	0	0.01	0.01	0	0
F	0	0	0	0	0	0	0	0	0	0	0.01	0	0	0
Me-1														
(N)	(38)	(37)	(17)	(30)	(49)	(2)	(22)	(27)	(40)	(80)	(33)	(48)	(30)	(35)
A	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	0	0	0	0	1.00
B	0	0	0	0	0	0	0	0	0	1.00	1.00	1.00	1.00	0
Me-2														
(N)	(38)	(37)	(17)	(30)	(49)	(2)	(22)	(27)	(40)	(80)	(33)	(48)	(30)	(35)
A	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	0	0	0	0	1.00
B	0	0	0	0	0	0	0	0	0	1.00	1.00	1.00	1.00	0
Mpi														
(N)	(38)	(34)	(16)	(29)	(42)	(2)	(22)	(27)	(40)	(67)	(32)	(44)	(30)	(35)
A	0	0.03	0	0.05	0	0	0	0	0.05	0	0	0	0.03	0
B	0.12	0.15	0.09	0.10	0.15	0	0.07	0.05	0.04	0.14	0.17	0.15	0.25	0
C	0.84	0.79	0.88	0.78	0.85	1.00	0.70	0.80	0.64	0.75	0.80	0.76	0.65	0.97
D	0.03	0	0	0.07	0	0	0.23	0.15	0.24	0.10	0.03	0.07	0.07	0.03
E	0.01	0.03	0.03	0	0	0	0	0	0.02	0.01	0	0.02	0	0
F	0	0	0	0	0	0	0	0	0.01	0	0	0	0	0
Pep-1														
(N)	(40)	(36)	(16)	(30)	(49)	(2)	(22)	(27)	(26)	(74)	(33)	(48)	(30)	(34)
A	0	0	0	0	0	0	0.09	0	0.29	0.01	0	0	0	0
B	1.00	1.00	1.00	1.00	0.98	1.00	0.89	0.98	0.65	0.99	1.00	1.00	1.00	1.00
C	0	0	0	0	0.02	0	0.02	0.02	0.06	0	0	0	0	0
Pep-2														
(N)	(40)	(37)	(17)	(30)	(49)	(2)	(22)	(27)	(16)	(80)	(33)	(48)	(30)	(35)
A	0	0	0	0	0	0	0	0	0	0	0	0	0	0.99
B	0	0	0.03	0	0	0	0	0	0	0	0	0	0	0.01
C	1.00	0.96	0.97	0.98	1.00	1.00	1.00	1.00	1.00	0	0	0	0	0
D	0	0.04	0	0.02	0	0	0	0	0	0	0	0	0	0
E	0	0	0	0	0	0	0	0	0	1.00	1.00	1.00	1.00	0

Table 2 (continued)

Locus	*P. subtilis* MI		*P. subtilis* MII				*P. paulensis*			*P. brasiliensis*				*P. schmitti*
	Atins	Fortaleza	Fortaleza	Recife	Ilhéus	Rio	Rio	Santos	L. Patos	Fortaleza	Ilhéus	Rio	Itajaí	Recife
Pgd														
(N)	(38)	(37)	(17)	(30)	(48)	(2)	(22)	(27)	(40)	(80)	(33)	(48)	(30)	(35)
A	1.00	1.00	0	0	0	0	0	0	0	0	0	0	0	0
B	0	0	0.76	0.67	0.62	0.50	0.07	0	0.01	0.02	0	0.01	0	1.00
C	0	0	0.24	0.33	0.38	0.50	0.93	1.00	0.98	0.98	1.00	0.98	1.00	0
D	0	0	0	0	0	0	0	0	0.01	0	0	0.01	0	0
Pgi														
(N)	(38)	(37)	(17)	(30)	(49)	(2)	(22)	(27)	(40)	(80)	(33)	(47)	(30)	(35)
A	0	0.01	0	0.02	0.01	0	0	0	0	0.01	0	0	0	0
B	0.60	0.56	0.94	0.70	0.91	0.50	0.84	0.89	0.99	0.95	0.98	0.95	0.97	1.00
C	0.38	0.42	0.06	0.28	0.08	0.25	0	0.11	0.01	0.04	0.02	0.01	0.03	0
D	0.02	0.01	0	0	0	0.25	0.16	0	0	0	0	0.04	0	0
Pgm-1														
(N)	(38)	(37)	(17)	(30)	(49)	(2)	(13)	(27)	(39)	(79)	(33)	(48)	(29)	(35)
A	0	0	0	0	0	0	0.19	0.11	0.01	0	0	0.01	0	0
B	0.04	0.07	0	0.02	0	0	0.12	0.11	0.05	0.01	0	0.02	0	0.14
C	0.96	0.93	1.00	0.98	1.00	1.00	0.69	0.78	0.94	0.99	1.00	0.97	1.00	0.83
D	0	0	0	0	0	0	0	0	0	0	0	0	0	0.03
Pgm-2														
(N)	(37)	(37)	(17)	(30)	(49)	(2)	(21)	(26)	(39)	(80)	(33)	(48)	(29)	(35)
A	0.01	0.03	0	0	0	0	0.12	0.10	0.05	0.01	0	0.01	0	0
B	0.95	0.95	1.00	0.98	0.99	1.00	0.88	0.88	0.95	0.95	0.97	0.97	0.97	0.99
C	0.04	0.02	0	0.02	0.01	0	0	0.02	0	0.04	0.03	0.02	0.03	0.01
H_o	0.10	0.11	0.08	0.08	0.06	0.07	0.10	0.07	0.09	0.04	0.03	0.04	0.04	0.03
H_e	0.10	0.13	0.08	0.10	0.07	0.07	0.12	0.09	0.10	0.05	0.04	0.05	0.05	0.03

deviations from Hardy–Weinberg expectations (heterozygote deficiencies, $P < 0.05$; Fisher's exact-test, corrected with Bonferroni series: Lessios 1992) were found for the *Pgm-1* locus in two populations of *Penaeus paulensis*. Heterozygote deficiencies are common in marine invertebrates (Hare et al. 1996), and could indicate population-mixing (the Wahlund effect: Wahlund 1928) or a number of actual or artifactual factors (for review see Zouros and Foltz 1984).

Heterozygosity levels (H) in the populations studied ($H = 0.03$ to 0.13; Table 2) were slightly higher than those observed by other authors in other species of *Penaeus* ($H = 0.006$ to 0.09: Mulley and Latter 1980; Lester 1983; Sunden and Davis 1991).

Two diagnostic loci (*Ak* and *Pgd*: Table 2) were found in the comparison of sympatric samples of the two morphotypes of *Penaeus subtilis* from Fortaleza (Ceará State). Furthermore, one diagnostic locus (*Mdh-1*: Table 2) was found between allopatric populations (Atins and Fortaleza) of *P. subtilis* MI. Pairwise values of unbiased genetic identity, I, and distance, D (Nei 1978), are given in Table 3. A tree showing genetic relatedness of all samples, based on UPGMA (Sneath and Sokal 1973) clustering of I values, is shown in Fig. 3.

Table 3 *Penaeus* spp. Unbiased gene identities, I (*above diagonal*) and unbiased genetic distances, D (*below diagonal*) (Nei 1978) of populations

Population	1	2	3	4	5	6	7	8	9	10	11	12	13	14
1. *P. subtilis* MI (Atins)		0.920	0.238	0.234	0.236	0.219	0.760	0.767	0.755	0.542	0.543	0.541	0.538	0.448
2. *P. subtilis* MI (Fortaleza)	0.083		0.149	0.140	0.145	0.127	0.838	0.844	0.830	0.614	0.614	0.614	0.612	0.327
3. *P. subtilis* MII (Fortaleza)	0.788	0.861		0.996	0.999	0.997	0.952	0.951	0.944	0.730	0.731	0.731	0.727	0.249
4. *P. subtilis* MII (Recife)	0.792	0.870	0.004		0.997	1.000	0.960	0.960	0.952	0.733	0.732	0.733	0.731	0.278
5. *P. subtilis* MII (Ilhéus)	0.789	0.865	0.001	0.003		1.000	0.967	0.967	0.961	0.744	0.745	0.744	0.742	0.272
6. *P. subtilis* MII (Rio)	0.803	0.881	0.003	0.000	0.000		0.981	0.980	0.967	0.748	0.749	0.750	0.744	0.287
7. *P. paulensis* (Rio)	0.274	0.177	0.050	0.040	0.034	0.019		0.999	0.992	0.278	0.277	0.276	0.279	0.365
8. *P. paulensis* (Santos)	0.265	0.170	0.050	0.040	0.034	0.020	0.001		0.990	0.266	0.265	0.265	0.268	0.360
9. *P. paulensis* (L. dos Patos)	0.281	0.187	0.057	0.050	0.040	0.034	0.008	0.010		0.275	0.274	0.275	0.275	0.378
10. *P. brasiliensis* (Fortaleza)	0.613	0.487	0.314	0.311	0.296	0.290	0.758	0.766	0.760		1.000	1.000	1.000	0.589
11. *P. brasiliensis* (Ilhéus)	0.612	0.487	0.313	0.312	0.294	0.290	0.758	0.767	0.760	0.000		1.000	1.000	0.586
12. *P. brasiliensis* (Rio)	0.615	0.487	0.314	0.311	0.295	0.288	0.759	0.767	0.760	0.000	0.000		1.000	0.590
13. *P. brasiliensis* (Itajaí)	0.620	0.491	0.319	0.314	0.298	0.296	0.757	0.765	0.759	0.000	0.000	0.000		0.601
14. *P. schmitti* (Recife)	0.639	0.721	0.780	0.757	0.762	0.751	0.694	0.697	0.685	0.555	0.556	0.555	0.548	

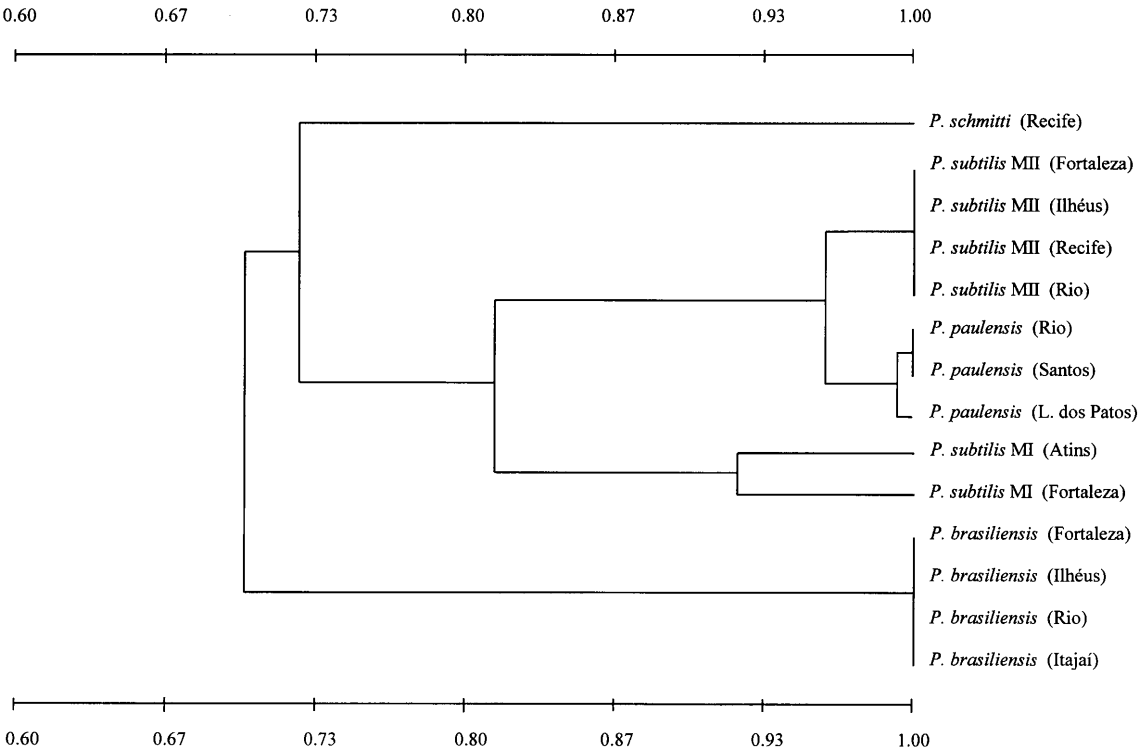

Fig. 3 *Penaeus* spp. Allozyme-based UPGMA similarity tree showing genetic relatedness (gene identity, *I*) of all samples

Sequencing

The partial sequences of the 3′ end of the CO1 gene analysed in the Brazilian penaeids (Fig. 4) were deposited in the GenBank under Accession Nos. AF248549 to AF248560. Using our sequence data and those from GenBank, pairwise levels of base divergence (*p* distances) between *Penaeus* species varied from 20.22% between *P. indicus* and *P. kerathurus* to 0.72% between the very closely related Western Atlantic species (possibly subspecies) *P. duorarum* and *P. notialis*. Mean nucleotide *p* distances were 5.5% between MI and MII of *P. subtilis* and 8.1% between these and *P. paulensis* (Table 4).

The highest level of intraspecific CO1 sequence divergence was 1.26% between two rare haplotypes of *Penaeus subtilis* MI. However, despite that difference, all *P. subtilis* MI samples grouped as a monophyletic cluster with high statistical support (Fig. 5). Intraspecific mean levels of sequence divergence (*p* distances) of the remaining species were 0.36, 0.14 and 0.27% for *P. subtilis* MII, *P. paulensis* and *P. brasiliensis*, respectively.

All Western Atlantic species, comprising sequences of both the putative subgenera *Farfantepenaeus* and *Litopenaeus*, grouped together in the neighbor-joining tree, forming a cluster with high statistical support (Fig. 5). This tree also provides further evidence of genetic divergence between the two morphotypes of *Penaeus subtilis*. Except for *P. canaliculatus*, *P. japonicus* and *P. kerathurus*, which clearly cluster together, relationships among the remaining Indo-Pacific species of *Penaeus* were not clearly defined.

PCR/RFLP analysis

Eight (A to H) restriction-based composite haplotypes were observed in the 178 samples analysed (Table 5). For each species, most individuals shared the same haplotype, with a few rare deviants (see Table 5). Haplotypic diversity of the studied penaeid species varied between 0 and 0.47 (Table 6). These values are smaller than that reported for a complete mitochondrial RFLP analysis of populations of the giant tiger shrimp *Penaeus monodon* (0.864: Klinbunga et al. 1999). This difference, however, was expected, since a larger set of restriction enzymes (11), and a longer DNA sequence were used to estimate haplotypic diversity of *P. monodon*. As the estimation of haplotype diversity is based solely on haplotype frequencies, it is sensitive to DNA size and the number of restriction enzymes used (Klinbunga et al. 1999). No haplotype was shared by any two individuals from different nominate species. Also, all individuals assigned to the two different *P. subtilis* morphotypes presented distinct composite haplotypes. One *AluI* site predicted from the sequences of all MII individuals was not detected by electrophoresis because the two fragments produced were too small (28 bp) to be visualised by ethidium bromide staining. Also the absence of the original 56 bp fragment was masked in MII patterns because of the presence of a fragment of similar size (54 bp) produced in the *AluI* MII restriction patterns. The revealed *AluI* site was only present in

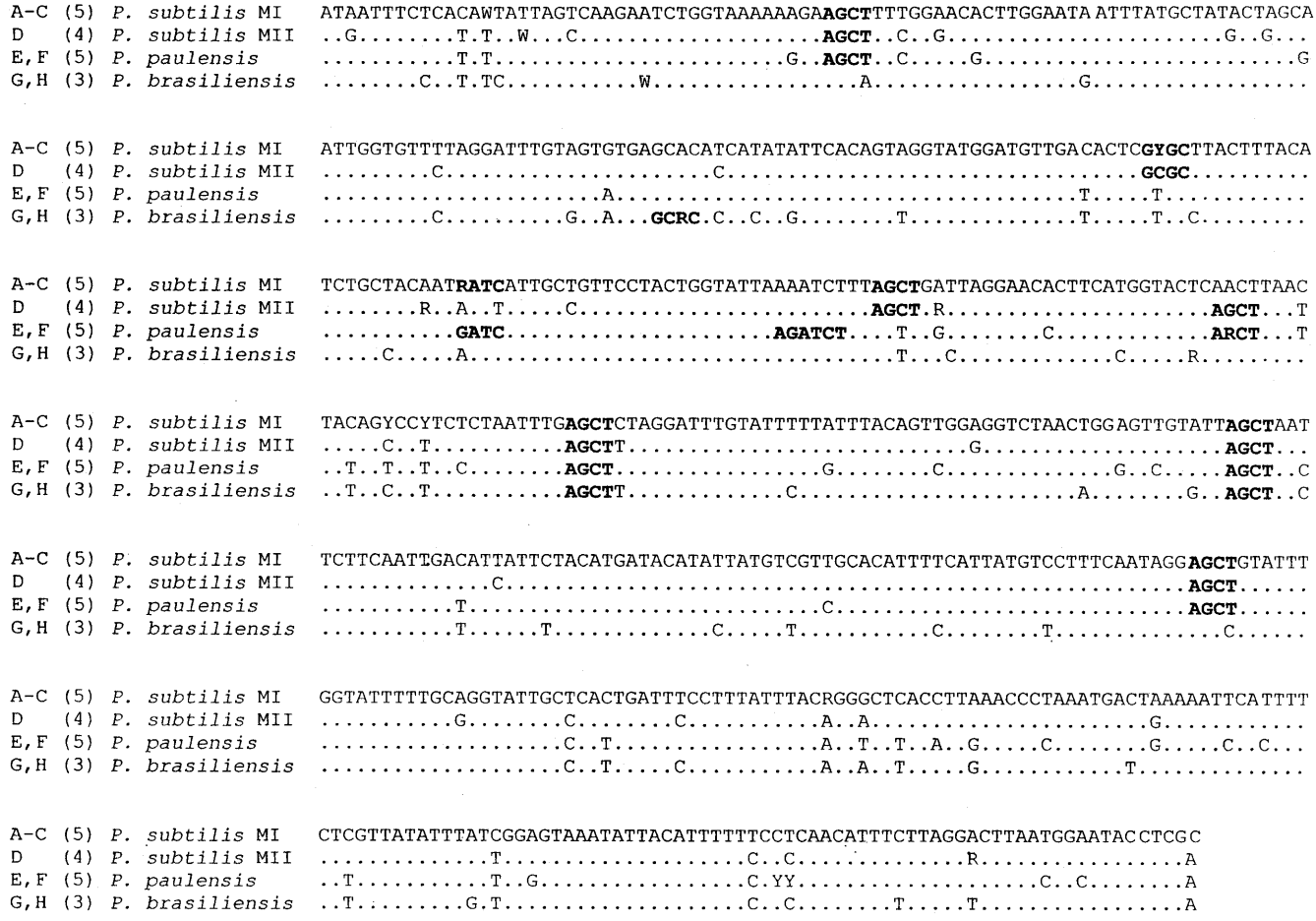

Fig. 4 *Penaeus* spp. Multiple-sequence alignment (CLUSTAL W 1.5) of partial sequences of CO1 gene (558 base pairs) from different Brazilian species. Restriction sites for polymerase chain-reaction/restriction-length polymorphism haplotypes are indicated as: *AluI* (AGCT); *HhaI* (GCGC); *BglII* (AGATCT); *NdeII* (GATC). *A – H* haplotypes found for each species; *numbers in parentheses* samples sequenced per species. Polymorphic sites within each species are indicated according to standard abbreviations [Nomenclature Committee of International Union of Biochemistry (NCIUB) 1985]: *R* = G or A, *Y* = C or T, *W* = A or T

individuals of MII, distinguishing samples of the two morphotypes.

Discussion

The allozyme and DNA-sequence data clearly show that the populations of *Penaeus subtilis* originally separated on the basis of small morphometric differences by Pérez Farfante (1969), and later named Morphotypes I and II by Maggioni (1996), are indeed distinct species as the latter author suggested. However, our results do not support the conspecificity of morphotype II of *P. subtilis* with *P. paulensis* (as suggested by Maggioni and by D'Incao et al. 1998). Conversely, the molecular analyses indicate the existence of a new species of *Penaeus*. Furthermore, our analyses of both nuclear and mitochondrial genes confirm the assertion of Baldwin et al. (1998) that the subgenera *Farfantepenaeus* and *Litopenaeus* should not be regarded as monophyletic units.

Species boundaries in Brazilian *Penaeus*

The presence of two diagnostic loci between the two morphotypes of *Penaeus subtilis* in sympatry clearly shows that the two morphotypes are not exchanging genes (i.e. they are not interbreeding) and, thus, cannot belong to the same biological species. Clear differences in total soluble proteins between those two morphotypes in Fortaleza and Natal were also found by Maggioni (1996). These differences were interpreted as indicative of species level differentiation, but the lack of differences between the banding patterns of *P. subtilis* MII and those of *P. paulensis* (from South Brazil) led Maggioni to conclude that the two were probably conspecific. Data from the isoelectric focusing of total proteins, however, are difficult to interpret genetically (Solé-Cava and Levy 1987), so the conclusions could only be preliminary (Maggioni 1996). Further work, using allozyme electrophoresis with populations from the same locality, confirmed that the sympatric Morphotypes I and II of *P. subtilis* were somewhat genetically divergent ($I = 0.947$), and that the latter was, indeed, more

Table 4 *Penaeus* spp. Kimura two-parameter (Kimura 1980; *below diagonal*) and *p* (Nei 1987; *above diagonal*) pairwise distances between haplotypes analysed (*A* – *H* haplotypes; nos. in parentheses individual sample number; *OTUs* operational taxonomic units)

OTUs	1	2	3	4	5	6	7	8	9	10
1. *P. canaliculatus*		0.1661	0.1606	0.1552	0.1661	0.1625	0.1949	0.1552	0.1588	0.1155
2. *P. monodon*	0.1895		0.1444	0.1390	0.1408	0.1372	0.1227	0.1390	0.1426	0.1480
3. *P. indicus*	0.1832	0.1624		0.1354	0.1498	0.1480	0.1444	0.1534	0.1498	0.1625
4. *P. semisulcatus*	0.1763	0.1568	0.1513		0.1408	0.1390	0.1372	0.1570	0.1372	0.1498
5. *P. duorarum*	0.1897	0.1572	0.1684	0.1575		0.0072	0.0993	0.1336	0.0939	0.1480
6. *P. notialis*	0.1848	0.1526	0.1660	0.1552	0.0073		0.0957	0.1300	0.0903	0.1480
7. *P. setiferus*	0.2294	0.1349	0.1619	0.1532	0.1084	0.1040		0.1191	0.1083	0.1625
8. *P. stylirostris*	0.1755	0.1555	0.1738	0.1786	0.1493	0.1447	0.1314		0.1047	0.1697
9. *P. vannamei*	0.1800	0.1599	0.1690	0.1529	0.1016	0.0973	0.1188	0.1141		0.1606
10. *P. japonicus*	0.1294	0.1662	0.1857	0.1691	0.1656	0.1656	0.1845	0.1950	0.1827	
11. *P. merguiensis*	0.1944	0.1647	0.1408	0.1441	0.1552	0.1529	0.1552	0.1884	0.1622	0.1825
12. *P. kerathurus*	0.1915	0.2119	0.2397	0.2022	0.1992	0.1942	0.2117	0.2126	0.2041	0.1845
13. *Sicyonia ingentis*	0.2257	0.1987	0.1916	0.2015	0.2175	0.2150	0.2106	0.2175	0.2251	0.2261
14. *P. subtilis* MI A (1–3)	0.2113	0.1486	0.1529	0.1697	0.1065	0.1022	0.0589	0.1401	0.1233	0.1773
15. *P. subtilis* MI B	0.2111	0.1507	0.1551	0.1671	0.1086	0.1042	0.0609	0.1423	0.1209	0.1772
16. *P. subtilis* MI C	0.2088	0.1417	0.1483	0.1602	0.1065	0.1022	0.0589	0.1333	0.1233	0.1773
17. *P. subtilis* MII D (1)	0.2090	0.1580	0.1624	0.1627	0.1111	0.1156	0.0839	0.1542	0.1257	0.1919
18. *P. subtilis* MII D (2,4)	0.2115	0.1604	0.1647	0.1651	0.1133	0.1178	0.0860	0.1566	0.1280	0.1944
19. *P. subtilis* MII D (3)	0.2113	0.1578	0.1622	0.1625	0.1132	0.1176	0.0837	0.1564	0.1278	0.1918
20. *P. paulensis* E	0.2218	0.1484	0.1763	0.1577	0.1380	0.1287	0.0836	0.1338	0.1344	0.2022
21. *P. paulensis* F (1,3–5)	0.2192	0.1507	0.1739	0.1553	0.1357	0.1265	0.0815	0.1315	0.1322	0.1997
22. *P. paulensis* F (2)	0.2192	0.1530	0.1739	0.1553	0.1357	0.1265	0.0836	0.1360	0.1322	0.1997
23. *P. brasiliensis* G	0.2038	0.1649	0.1762	0.1552	0.1065	0.1022	0.0917	0.1382	0.1144	0.1890
24. *P. brasiliensis* H (1)	0.2061	0.1671	0.1784	0.1574	0.1086	0.1042	0.0958	0.1380	0.1187	0.1889
25. *P. brasiliensis* H (2,3)	0.2038	0.1649	0.1762	0.1552	0.1087	0.1043	0.0938	0.1359	0.1166	0.1890

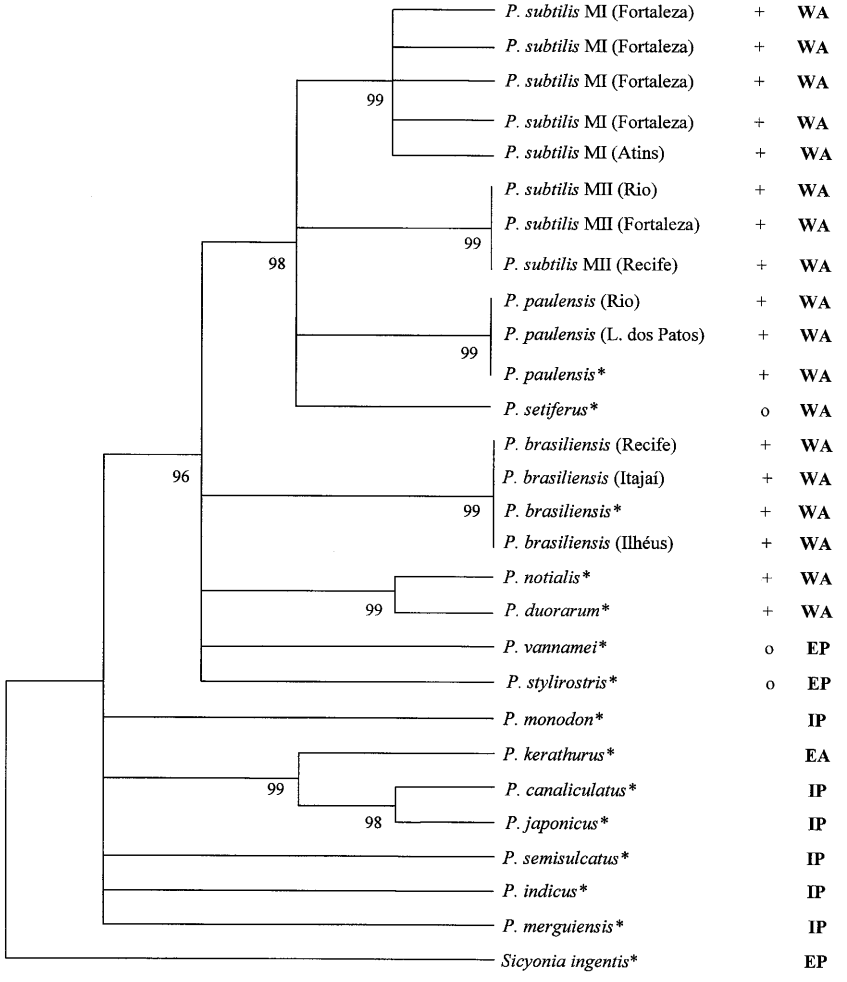

Fig. 5 *Penaeus* spp. Cytochrome oxidase 1-based neighbor-joining tree: *numbers under branches* Student's *t*-test (confidence probability) of branch lengths; *Sequences taken from GenBank; + *Farfantepenaeus* spp.; o *Litopenaeus* spp.; *EA* Eastern Atlantic; *EP* Eastern Pacific Ocean; *IP* Indo-Pacific; *WA* Western Atlantic

Table 4 (continued)

11	12	13	14	15	16	17	18	19	20	21	22	23	24	25
0.1697	0.1661	0.1931	0.1823	0.1823	0.1805	0.1805	0.1823	0.1823	0.1895	0.1877	0.1877	0.1769	0.1787	0.1769
0.1462	0.1823	0.1733	0.1336	0.1354	0.1282	0.1408	0.1426	0.1408	0.1336	0.1354	0.1372	0.1462	0.1480	0.1462
0.1264	0.2022	0.1679	0.1372	0.1390	0.1336	0.1444	0.1462	0.1444	0.1552	0.1534	0.1534	0.1552	0.1570	0.1552
0.1300	0.1751	0.1751	0.1498	0.1480	0.1426	0.1444	0.1462	0.1444	0.1408	0.1390	0.1390	0.1390	0.1408	0.1390
0.1390	0.1733	0.1877	0.0975	0.0993	0.0975	0.1011	0.1029	0.1029	0.1227	0.1209	0.1209	0.0975	0.0993	0.0993
0.1372	0.1697	0.1859	0.0939	0.0957	0.0939	0.1047	0.1065	0.1065	0.1155	0.1137	0.1137	0.0939	0.0957	0.0957
0.1390	0.1823	0.1823	0.0560	0.0578	0.0560	0.0776	0.0794	0.0776	0.0776	0.0758	0.0776	0.0848	0.0884	0.0866
0.1643	0.1823	0.1877	0.1264	0.1282	0.1209	0.1372	0.1390	0.1390	0.1209	0.1191	0.1227	0.1245	0.1245	0.1227
0.1444	0.1769	0.1931	0.1119	0.1101	0.1119	0.1137	0.1155	0.1155	0.1209	0.1191	0.1191	0.1047	0.1083	0.1065
0.1606	0.1606	0.1931	0.1570	0.1570	0.1570	0.1679	0.1697	0.1679	0.1751	0.1733	0.1733	0.1661	0.1661	0.1661
	0.1805	0.1733	0.1462	0.1444	0.1444	0.1480	0.1498	0.1480	0.1516	0.1534	0.1570	0.1300	0.1336	0.1318
0.2086		0.2058	0.1715	0.1715	0.1697	0.1805	0.1823	0.1823	0.1949	0.1931	0.1931	0.1661	0.1697	0.1679
0.1978	0.2445		0.1859	0.1895	0.1859	0.1913	0.1931	0.1913	0.1841	0.1823	0.1859	0.1859	0.1859	0.1859
0.1649	0.1967	0.2160		0.0054	0.0072	0.0542	0.0560	0.0542	0.0758	0.0740	0.0776	0.0921	0.0957	0.0939
0.1624	0.1965	0.2209	0.0054		0.0126	0.0596	0.0614	0.0596	0.0776	0.0758	0.0794	0.0903	0.0939	0.0921
0.1625	0.1942	0.2160	0.0073	0.0128		0.0505	0.0523	0.0505	0.0722	0.0704	0.0740	0.0884	0.0921	0.0903
0.1675	0.2094	0.2235	0.0572	0.0632	0.0532		0.0018	0.0072	0.0848	0.0866	0.0866	0.0939	0.0975	0.0957
0.1699	0.2119	0.2261	0.0592	0.0652	0.0552	0.0018		0.0054	0.0866	0.0884	0.0884	0.0957	0.0993	0.0975
0.1673	0.2117	0.2233	0.0571	0.0631	0.0531	0.0073	0.0054		0.0884	0.0903	0.0903	0.0957	0.0993	0.0975
0.1715	0.2307	0.2137	0.0815	0.0835	0.0773	0.0923	0.0944	0.0964		0.0018	0.0054	0.1011	0.1047	0.1029
0.1739	0.2280	0.2111	0.0794	0.0813	0.0752	0.0944	0.0966	0.0986	0.0018		0.0036	0.0993	0.1029	0.1011
0.1788	0.2280	0.2162	0.0836	0.0856	0.0794	0.0944	0.0966	0.0986	0.0054	0.0036		0.0993	0.1029	0.1011
0.1440	0.1897	0.2153	0.1006	0.0983	0.0963	0.1030	0.1052	0.1050	0.1111	0.1089	0.1089		0.0054	0.0036
0.1484	0.1944	0.2151	0.1048	0.1025	0.1005	0.1072	0.1094	0.1092	0.1154	0.1132	0.1132	0.0054		0.0018
0.1463	0.1921	0.2153	0.1028	0.1005	0.0984	0.1052	0.1074	0.1072	0.1133	0.1111	0.1111	0.0036	0.0018	

Table 5 *Penaeus* spp. PCR/RFLP (polymerase chain-reaction/restriction-length polymorphism) composite haplotypes observed for each species and sampling site (*N* no. of individuals analysed)

Sampling site	Sample (*N*)	Composite haplotypes							
		A	B	C	D	E	F	G	H
Atins	*P. subtilis* MI (4)	4	–	–	–	–	–	–	–
Fortaleza	*P. subtilis* MI (18)	15	1	2	–	–	–	–	–
	P. subtilis MII (16)	–	–	–	16	–	–	–	–
	P. brasiliensis (8)	–	–	–	–	–	–	3	5
Recife	*P. subtilis* MII (20)	–	–	–	20	–	–	–	–
	P. brasiliensis (2)	–	–	–	–	–	–	2	–
Ilhéus	*P. subtilis* MII (13)	–	–	–	13	–	–	–	–
	P. brasiliensis (7)	–	–	–	–	–	–	1	6
Rio	*P. subtilis* MII (2)	–	–	–	2	–	–	–	–
	P. paulensis (21)	–	–	–	–	1	20	–	–
	P. brasiliensis (28)	–	–	–	–	–	–	11	17
Santos	*P. paulensis* (15)	–	–	–	–	–	15	–	–
Itajaí	*P. brasiliensis* (11)	–	–	–	–	–	–	3	8
Lagoa dos Patos	*P. paulensis* (13)	–	–	–	–	–	13	–	–
Total	*P. subtilis* MI (22)	19	1	2	–	–	–	–	–
	P. subtilis MII (51)	–	–	–	51	–	–	–	–
	P. paulensis (49)	–	–	–	–	1	48	–	–
	P. brasiliensis (56)	–	–	–	–	–	–	20	36

Table 6 *Penaeus* spp. Haplotypic diversity of PCR/RFLP data [*N* no. of individuals analysed; *S* no. of samples analysed; *h* Nucleon diversity index (Nei 1987)]

Species	*N*	*S*	Haplotypes	*h*
P. subtilis MI	22	2	3	0.25
P. subtilis MII	51	4	1	0.00
P. paulensis	49	3	2	0.04
P. brasiliensis	56	5	2	0.47

closely related to *P. paulensis* ($I = 0.985$: D'Incao et al. 1998). The genetic identity observed between the two morphs of *P. subtilis* by D'Incao et al. was high in relation to the values usually found between congeneric species (Hedgecock et al. 1982; Thorpe 1982; Thorpe and Solé-Cava 1994). However, the presence of one diagnostic locus (*Pgd*) between the two morphotypes in sympatry, regardless of their high similarity, demonstrated that they were not exchanging genes and there-

fore could not belong to the same species (see e.g. Solé-Cava et al. 1985). Because of their high similarity, they should, rather, be classified as a pair of distinct sibling biological species (sensu Knowlton 1986). In this study, we found one further diagnostic locus (*Ak*) between the two morphs of *P. subtilis*, lowering the genetic identity between them ($I = 0.788$ to 0.881) to a value more typical of interspecific comparisons (Thorpe and Solé-Cava 1994). Moreover, the genetic differentiation observed between these two species was stable (Fig. 3), even over a considerable geographic distance (2700 km), confirming that each comprises an independent evolutionary unit.

The high allozyme similarity observed by D'Incao et al. (1998) between *Penaeus subtilis* MII and *P. paulensis* from a single locality led them to wrongly conclude that these species were conspecific. In our analysis, we also found a high similarity between *P. subtilis* MII and *P. paulensis* ($I = 0.944$ to 0.981), and no diagnostic loci (sensu Ayala 1983) could be found between them (Table 2). However, the analysis of several populations in this study showed that, regardless of the large geographic area sampled, each species appeared as a monophyletic group (Fig. 3).

The CO1 sequences of MII were also very distinct (8.48 to 9.03% nucleotide divergence – *p* distance) from those of *Penaeus paulensis*, and also, to a lesser extent, from those of *P. subtilis* MI (5.05 to 6.14%) (Table 4). Furthermore, no shared RFLP haplotypes were found in an analysis of 122 individuals of different samples of the three species (Table 5), once again indicating their evolutionary independence. It is thus clear that the two morphotypes of *P. subtilis* are genetically different from each other and also from *P. paulensis*, despite their high morphological resemblance. Sequence analyses of mtDNA gene fragments have already revealed that morphological similarity may mask divergent evolution in penaeids (Palumbi and Benzie 1991).

One diagnostic locus (*Mdh-1*) was found between populations of *Penaeus subtilis* MI from Atins and Fortaleza. This difference lowered the estimated gene identity between those two populations to 0.92, still well within the normally accepted level of intraspecific differentiation (Thorpe 1982), but nonetheless lower than the average gene identity found between the populations of *P. subtilis* II and *P. paulensis* ($I = 0.95$; Fig. 3). This difference alone cannot be regarded as direct proof of species-level differentiation, because the populations are allopatric (see Thorpe and Solé-Cava 1994). Furthermore, levels of sequence divergence between individuals of those two populations were very low ($p = 0.0000$ to 0.0072), and they appear as a monophyletic cluster in both allozyme and CO1 trees. It would be interesting to investigate populations of *P. subtilis* MI between Fortaleza and Atins and further north to verify whether there is a strong population boundary between the two regions.

The currently accepted distribution of *Penaeus subtilis* runs from the Caribbean south to Rio de Janeiro (Pérez Farfante 1969). The molecular analyses indicate that the species corresponding to MII can be found at least from Fortaleza (Ceará; 03°44′S; 38°31′W) to Cabo Frio (Rio de Janeiro; 22°53′S; 42°02′W), while that corresponding to *P. subtilis* MI was not found, in the present work, in any of the sample sites studied south of Fortaleza. These results strongly indicate that the currently accepted distribution of *P. subtilis* in South America is the result of the sum of the distribution of two different species, with a narrow overlap zone in northeast Brazil (see Fig. 1).

Timing of divergences and geological context

The levels of CO1 sequence divergence found between the different Brazilian species of *Penaeus* were similar to those observed between species of the snapping shrimp *Alpheus* (Knowlton et al. 1993; Knowlton and Weigt 1998). Using the calibration for mangrove species of *Alpheus* from the Atlantic and Pacific sides of Panamá, we conclude that *P. subtilis* Morphotypes I and II have probably diverged around 4.2 million years before present (mybp). Likewise, the ancestral lineage of those two species diverged from that of *P. paulensis* about 6.3 mybp.

Profound modifications have occurred in the geology of the Amazonian region since the Miocene, and these may have had important effects on the coastal marine fauna (Lovejoy et al. 1998). In the late Miocene and early Pliocene (7 to 4 mybp), substantial Andean uplift led to major changes in paleogeography, and the Andes and their drainages attained their present configuration (Hoorn 1994; Hoorn et al. 1995). During this process, the Orinoco changed its course, the Amazon Atlantic drainage was established, and the Amazon–Caribbean connection was closed (Hoorn et al. 1995). During the last 4 million years, extensive sea-level oscillations have occurred. Associated with transgression periods, in hotter and moister periods, the overall water discharge of the whole Amazon basin, including the melting of Andean ice, may have resulted in the recurrent formation of a huge "Amazon lagoon" (Klammer 1984; Frailey et al. 1988). Several biogeographic patterns are in accordance with the Amazon lagoon hypothesis (Marroig and Cerqueira 1997). Since the development of penaeid post larvae and juveniles is associated with estuarine environments and influenced by environmental conditions such as salinity and temperature (Gunter et al. 1964; Pérez Farfante 1969), the changing of discharge patterns, sea (and lagoon) level and river out-flow levels could have led to disturbances in the distribution of penaeid species, and may have led to speciation.

Systematic status of subgenera

Our results agree with those of Baldwin et al. (1998), in that subgenera based on the morphology of the female

thelycum are not supported by molecular data. In the present phylogenetic reconstruction, the Western Atlantic species belonging to the subgenera *Farfantepenaeus* (closed thelycum) and *Litopenaeus* (open thelycum) group together with the eastern Pacific *Litopenaeus* species in a polyphyletic cluster. Furthermore, in the allozyme analysis (Table 3; Fig. 3), *Penaeus* (*Farfantepenaeus*) *brasiliensis* was left out of the group formed by *P.* (*F.*) *subtilis*, *P.* (*F.*) *paulensis*, *P.* (*Litopenaeus*) *schmitti*, once again indicating paraphyly. Therefore, the molecular data (both allozymes and mitochondrial DNA) indicate that the open thelycum has evolved independently several times within the penaeids, and that the separation of the two putative subgenera is not justified.

Fisheries implications

The present work revealed a new species of *Penaeus*, using allozyme and cytochrome oxidase 1 analyses. The implications of these findings for *Penaeus* fisheries along the north and northeast regions of Brazil are obviously important, and a revaluation of the management of these resources is necessary. Penaeids are fished in the Southwest Atlantic both at the juvenile and adult stages (Valentini et al. 1991). There is evidence of declining stocks along the Brazilian coast: Brazilian shellfish exports fell from ~11 000 metric tonnes in 1994 to <5 000 metric tonnes in 1997 (FAO 1999). The decline in stocks is probably a result of an increased number of small boats fishing in natural nurseries combined with earlier unrestricted growth of the industrial fleet, possibly through inefficient legislation and inadequate supervision of the catches (D'Incao 1991; Valentini et al. 1991). For management purposes, *P. subtilis* should not continue to be treated as a single species. Whilst MII populations seem to occur all along the eastern Brazilian coast, populations of MI possibly have a more limited distribution, occurring on the fishing grounds of at least part of northeast Brazil.

One important use of molecular markers for the study of marine invertebrate fisheries is the correct species-level identification of individuals at different developmental stages (Thorpe et al. 2000). The RFLP analysis of the CO1 gene has produced species-specific markers that will be useful as an auxiliary tool for the identification of larvae and commercial products of each species. These markers should also be helpful in the determination of the origins of nursery stocks and species distributions, and represent the first development of molecular tools for the supervision and management of Southwest Atlantic shrimp fisheries.

Acknowledgements The authors would like to thank E. Araújo, F. D'Incao, L. Weber, P. Paiva and P. Vianna for help with collecting the samples, F. D'Incao for the initial identification of the two morphotypes of *Penaeus subtilis*, G. Solha for technical assistance and C. Russo for help with the phylogenetic analysis of CO1 sequences. Our thanks also to S. Williams and the Smithsonian Institution for the DNA-sequencing of some of the samples, and to N. Knowlton for critical comments on the manuscript. This work was supported by grants from CAPES, CNPq, FAPERJ, FUJB and PADCT (Brazil).

References

Ayala FJ (1983) Enzymes as taxonomic characters. In: Oxford GS, Rollinson D (eds) Protein polymorphism: adaptive and taxonomic significance. Academic Press, London, pp 3–26

Baldwin JD, Bass AL, Bowen BW, Clark WH Jr (1998) Molecular phylogeny and biogeography of the marine shrimp *Penaeus*. Mol Phylogenetics Evolut 10: 399–407

Brewer GJ (1970) An introduction to isozyme techniques. Academic Press, New York

Chow S, Clarke ME, Walsh PJ (1993) PCR-RFLP analysis on thirteen western Atlantic snappers (subfamily Lutjaninae): a simple method for species and stock identification. Fish Bull US 91: 619–627

D'Incao F (1991) Pesca e biologia de *Penaeus paulensis* na Lagoa dos Patos, RS. Atlântica, Rio Grande 13: 159–169

D'Incao F, Delevedove G, Maggioni DE, Maggioni R (1998) Evidência genética da presença de *Farfantepenaeus paulensis* (Pérez Farfante, 1967) no litoral nordeste do Brasil (Decapoda: Penaeidae). Nauplius 6: 129–137

Fabricius JC (1798) Suplementum entomologiae systematicae. Hafniae

FAO (1999) Fisheries series 53 (Statistics series 149). FAO, Rome

Frailey CD, Lavina EL, Rancy A, Filho JPS (1988) A proposed Pleistocene/Holocene lake in the Amazon basin and its significance to Amazonian geology and biogeography. Acta amazonica 18: 119–143

Garcia DK, Benzie JAH (1995) RAPD markers of potential use in penaeid prawn (*Penaeus monodon*) breeding programs. Aquaculture, Amsterdam 130: 137–144

Gunter G, Christmas JY, Killebrew R (1964) Some relations of salinity to population distributions of motile estuarine organisms, with special reference to penaeid shrimp. Ecology 45: 181–185

Hare MP, Karl SA, Avise JC (1996) Anonymous nuclear DNA markers in the American oyster and their implications for the heterozygote deficiency phenomenon in marine bivalves. Molec Biol Evolut 13: 334–345

Harris H, Hopkinson DA (1978) Handbook of enzyme electrophoresis in human genetics. North Holland, Amsterdam

Hedgecock D, Tracey ML, Nelson K (1982) Genetics. In: Bliss D (ed) The biology of Crustacea. Vol. 2. Academic Press, New York, pp 283–403

Hoelzel AR, Green A (1992) Analysis of population-level variation by sequencing PCR-amplified DNA. In: Hoelzel AR (ed) Molecular genetic analysis of populations. Oxford University Press, Oxford, pp 159–188

Hoorn C (1994) An environmental reconstruction of the Palaeo–Amazon River system (Middle–Late Miocene, NW Amazonia). Palaeogeogr Palaeoclim Palaeoecol 112: 187–238

Hoorn C, Guerrero J, Sarmiento GA, Lorente MA (1995) Andean tectonics as a cause for changing drainage patterns in Miocene northern South America. Geology 23: 237–240

Kimura M (1980) A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. J Molec Evolut 16: 111–120

Klammer G (1984) The relief of the extra-Andean Amazon basin. In: Sioli H (ed) The Amazon: limnology and landscape ecology of a mighty tropical river and its basin. W Junk Publishers, Dordrecht, pp 47–83

Klinbunga S, Penman DJ, McAndrew BJ, Tassanakajon A (1999) Mitochondrial DNA diversity in three populations of the giant tiger shrimp *Penaeus monodon*. Mar Biotechnol 1: 113–121

Knowlton N (1986) Cryptic and sibling species among the decapod crustacea. J Crustacean Biol 6: 356–363

Knowlton N, Weigt LA (1998) New dates and new rates for divergence across the Isthmus of Panama. Proc R Soc 265: 2257–2263

Knowlton N, Weigt LA, Solórzano LA, Mills DK, Bermingham E (1993) Divergence in proteins, mitochondrial DNA, and reproductive compatibility across the Isthmus of Panama. Science, NY 260: 1629–1632

Kumar S, Tamura K, Nei M (1994) MEGA: molecular evolutionary genetics analysis software for microcomputers. Cabios 10: 189–191

Lessios HA (1992) Testing electrophoretic data for agreement with Hardy–Weinberg expectations. Mar Biol 112: 517–523

Lester LJ (1983) Developing a selective breeding program for penaeid shrimp mariculture. Aquaculture, Amsterdam 33: 41–50

Lovejoy NR, Bermingham E, Martin AP (1998) Marine incursion into South America. Nature, Lond 396: 421–422

Maggioni DE (1996) Caracterização de algumas espécies do gênero *Penaeus* do litoral brasileiro através de eletroenfoque. Nauplius 4: 129–137

Manchenko GP (1994) Handbook of detection of enzymes on electrophoretic gels. CRC Press, Ann Arbor, Michigan

Marroig G, Cerqueira R (1997) Plio–Pleistocene South American history and the Amazon lagoon hypothesis: a piece in the puzzle of Amazonian diversification. J comp Biol 2: 103–119

Mulley JC, Latter BDH (1980) Genetic variation and evolutionary relationships within a group of thirteen species of penaeid prawns. Evolution 34: 904–916

Murphy RW, Sites JW, Buth DG, Haufler CH (1990) Proteins. I. Isozyme electrophoresis. In: Hillis DM, Moritz C (eds) Molecular systematics. Sinauer Associates, Massachusetts, pp 45–126

Nei M (1978) Estimation of average heterozygosity and genetic distance from a small number of individuals. Genetics, Austin, Tex 89: 583–590

Nei M (1987) Molecular evolutionary genetics. Columbia University Press, New York

Nomenclature Committee of IUB (NCIUB) and IUPAC-IUB Joint Commission on Biochemical Nomenclature (JCNB) (1985) Biological chemistry Hoppe-Seyler. Walter de Grayter, Berlin, pp 3–7

Palumbi RS, Benzie J (1991) Large mitochondrial DNA differences between morphologically similar penaeid shrimp. Molec mar Biol Biotechnol 1: 27–34

Pérez Farfante I (1967) A new species and two new subspecies of shrimp of the genus *Penaeus* from the Western Atlantic. Proc biol Soc Wash 80: 83–100

Pérez Farfante I (1969) Western Atlantic shrimp of genus *Penaeus*. Fish Bull US 67: 461–591

Pérez Farfante I, Kensley B (1997) Penaeoid and sergestoid shrimps and prawns of the world. Keys and diagnoses for the families and genera. Mém Mus natn Hist Nat, Paris 175: 1–79

Rzhetsky A, Nei M (1992) A simple method for estimating and testing minimum-evolution trees. Molec Biol Evolut 9: 945–967

Saitou N, Nei M (1987) The neighbor-joining method: a new method for reconstructing phylogenetic trees. Molec Biol Evolut 4: 406–425

Sambrook J, Fritsch EF, Maniatis T (1989) Molecular cloning – a laboratory manual. Cold Spring Harbor Laboratory Press, New York

Sanger F, Nicklen S, Coulson AR (1977) DNA sequencing with chain-terminating inhibitors. Proc natn Acad Sci USA 74: 5463–5467

Shaw CR, Prasad R (1970) Starch gel electrophoresis of enzymes – a compilation of recipes. Biochem Genet 4: 297–320

Sneath PHA, Sokal RR (1973) Numerical taxonomy the principles and practice of numerical classification W. H. Freeman Co., San Francisco

Solé-Cava AM, Levy JA (1987) Biochemical evidence for a third species of angel shark (*Squatina*) off the east coast of South America. Biochem Syst Ecol 15: 135–144

Solé-Cava AM, Thorpe JP, Kaye JG (1985) Reproductive isolation with little genetic divergence between *Urticina* (= *Tealia*) *felina* and *U. eques* (Anthozoa: Actiniaria). Mar Biol 85: 279–284

Sunden SLF, Davis SK (1991) Evaluation of genetic variation in a domestic population of *Penaeus vannamei* (Boone): a comparison with three natural populations. Aquaculture, Amsterdam 97: 131–142

Swofford DL, Selander RB (1981) BIOSYS-1, a FORTRAN programme for the comprehensive analysis of electrophoretic data in population genetics and systematics. J Hered 72: 281–283

Thompson JD, Higgins DG, Gibson TJ (1994) CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Res 22: 4673–4680

Thorpe JP (1982) The molecular clock hypothesis: biochemical evolution, genetic differentiation and systematics. A Rev Ecol Syst 13: 139–168

Thorpe JP, Solé-Cava AM (1994) The use of allozyme electrophoresis in invertebrate systematics. Zoologica Scr 23: 3–18

Thorpe JP, Solé-Cava AM, Watts PC (2000) Exploited marine invertebrates: genetics and fisheries. In: Solé-Cava AM, Russo CAM, Thorpe JP (eds) Marine genetics. Kluwer Academic Publishers, Dordrecth, pp 165–184

Valentini H, D'Incao F, Rodrigues LF, Neto JER, Rahn E (1991) Análise da pesca do camarão-rosa (*Penaeus brasiliensis e Penaeus paulensis*) nas regiões sudeste e sul do Brasil. Atlântica, Rio Grande 13: 143–157

Wahlund S (1928) Zusammensetzung von Populationen und Korrelationserscheinungen vom Standpunkt der Vererbungslehre aus betrachtet. Hereditas 11: 65–105

Ward RD, Beardmore JA (1977) Protein variation in the plaice (*Pleuronectes platessa*). Genet Res 30: 45–62

Zouros E, Foltz DW (1984) Possible explanations of heterozygote deficiency in bivalve molluscs. Malacologia 25: 583–591