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Phylogenetic Relationships of 12 Penaeoidea Shrimp Species Deduced from Mitochondrial DNA Sequences

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*DNA sequences of an 847 bp fragment of mitochondrial cytochrome oxidase subunit I (COI) gene and a 514 bp fragment of 16s rRNA gene were determined to examine the phylogenetic relationships of 12 Penaeoidea shrimp species (*Penaeus chinensis, Penaeus japonicus, Penaeus penicillatus, Penaeus vannamei, Penaeus canaliculatus, Trachypenaeus curvirostris, Metapenaeus affinis, Metapenaeus ensis, Metapenaeopsis barbata, Parapenaeus fissuroides, Parapenaeopsis hardiwickii, Solenocera crassicornis*). Both fragments of the swimming crab* Portunus trituberculaus *chosen as the outgroup were also sequenced. Intraspecific sequence divergence of 0.24–1.2% in the COI gene was found in 5 species, while no intraspecific variation was observed in the 16s rRNA gene. Three phylogenetic trees based on the 1361 bp combined sequences of COI and 16s rRNA were concordant in indicating the following suggestions: (1) phylogenetic relationship of the 11 Penaeidae species based on our result support the opinion of Burkenroad (Burkenroad, M. D. (1983).* Crustacean Issues ***3****:279–290) on the basis of morphological features; (2) it seems more reasonable to class* Solenocera crassicorni *in the family Penaeidae; (3) the fragment of the COI gene chosen here appears to be a good*

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marker for speciation studies and population analysis in Crustaceans, while the 16s rRNA gene fragment here seems suitable for examining phylogenetic relationships at the species or genus levels in Crustaceans. Our time estimates suggest that Penaeus *and* Metapenaeus *might have separated about* 6.38×10^6–7.98×10^6 *years BP in the post-Miocene, and the species separation within* Metapenaeus *and* Penaeus *might occur* 0.08×10^6–0.4×10^6 *years BP in the late Pleistocene.*

KEY WORDS: molecular phylogeny; mtDNA; COI gene; 16s rRNA gene; Penaeoidea.

INTRODUCTION

Marine shrimps of the superfamily Penaeoidea represent approximately one third of the world's commercially important shrimp species and account for over 80% of the wild catch (Baldwin *et al*., 1998). In China, Penaeoidea shrimps are of economic importance in both fishing and seafarming industries. As noted by Bernatchez (1995), wildlife management strategies are contingent on an understanding of the evolutionary underpinning of contemporary biodiversity. In this case, phylogenetic information can be used to guide captive breeding programs that are intended to produce superior captive strains through hybridization of closely related species. Unfortunately, recent studies indicate that some taxonomic relationships based solely on morphology, to some extent, cannot reflect the real evolutionary relationship. For instance, Palumbi and Benzie (1991) found surprisingly high genetic differentiation (10%) between two morphologically and ecologically similar *Penaeus* species. The analysis of COI variation in *Penaeus* shrimp also indicated that current subgenera assignments do not reflect evolutionary partitions within the genus *Penaeus* (Baldwin *et al*., 1998). Thus, DNA sequence information from some more species in the family Penaeoidea may shed light on the evolutionary relationships of these shrimps.

Previous studies suggested that the mitochondrial cytochrome oxidase subunit I (COI) gene was useful in examining taxonomic and phylogenetic relationships in many arthropods (Crozier *et al*., 1989; Garcia-Machado *et al*., 1993; Howland and Hewitt, 1995; Navajas *et al*., 1998, 1999; Satta and Takahata, 1990; Spicer, 1995). In terms of marine crustaceans, the COI gene has been used to analyze phylogenetic relationships of many taxa including 13 species in *Penaeus*, 6 species in *Gammarus*, 9 species in *Cancer*, and Bresiliidae shrimps (Baldwin *et al*., 1998; Harrison and Crespi, 1999; Meyran *et al*., 1997; Palumbi and Benzie, 1991; Shank *et al*., 1999). The 16s rRNA gene was also applied to study the phylogeny of Penaeid shrimp species, Porcelain crabs, Grapsoid crabs, and Ocypodid crabs (Kitaura *et al*., 1998; Maggioni *et al*., 2001; Schubart *et al*., 2000; Stillman and Reeb, 2001). In this study, therefore, we sequenced COI and 16s rRNA genes of 12 economically important species in Penaeoidea mainly distributed in the Chinese coast in order to investigate the genetic differentiation and phylogenetic relationships among them.

MATERIALS AND METHODS

Sampling

The species investigated in this study are as follows: *Penaeus chinensis*, *Penaeus japonicus*, *Penaeus penicillatus*, *Penaeus vannamei*, *Penaeus canaliculatus*, *Trachypenaeus curvirostris*, *Metapenaeus affinis*, *Metapenaeus ensis*, *Metapenaeopsis barbata*, *Parapenaeus fissuroides, Parapenaeopsis hardiwickii*, and *Solenocera crassicornis*. Table I summarizes the collection sites and numbers of these samples. Swimming crab *Portunus trituberculatus* was chosen as an outgroup.

Table I. Collection Locations of 12 Marine Shrimp Species

Taxonomic designation	Specimen identification	Number of specimens	Collection location
Penaeidae			
Penaeus japonicus	Pjap	2	Coast of Shenzhen in the South China Sea
Penaeus penicillatus	Ppen	2	Coast of Xiamen in the East China Sea
Penaeus chinensis	Pchi01–Pchi06	6	West coast of Korea Peninsula
	Pchi07–Pchi12	6	Central waters of the Yellow Sea
	Pchi13–Pchi18	6	North coast of the Shandong Peninsula in the Bohai Sea
	Pchi19–Pchi24	6	South coast of the Shandong Peninsula in the Yellow Sea
Penaeus vannamei	Pvan	1	Aquaculture farm in Qingdao, China
Penaeus canaliculatus	Pcan	1	Coast of Shenzhen in the South China Sea
Trachypenaeus curvirostris	Tcur	1	Coast of Qingdao in the Yellow Sea
Metapenaeus affinis	Maff	4	Coast of Xiamen in the East China Sea
Metapenaeus ensis	Mens	2	Coast of Xiamen in the East China Sea
Metapenaeopsis barbata	Mbar	1	Coast of Shenzhen in the South China Sea
Parapenaeus fissuroides	Pfis	1	Coast of Zhoushan in the East China Sea
Parapenaeopsis hardiwickii	Phar	1	Coast of Zhoushan in the East China Sea
Solenoceridae			
Solenocera crassicornis	Scra	1	Coast of Zhoushan in the East China Sea
Portunidae			
Portunus trituberculatus	Ptris	1	Coast of Qingdao in the Yellow Sea

Species Identification

Species were morphologically recognized according to the taxonomic criteria cited in the books *The Biology of the Penaeidae* (Dall *et al*., 1990) and *Penaeoid Shrimps of the South China Sea* (Liu and Zhang, 1986).

Genomic DNA Extraction

Genomic DNA was extracted from about 50 mg of muscle tissue following the protocols of Zhang and Ryder (1994) and then stored at −70°C.

PCR Amplification

Two target DNA fragments were amplified and sequenced: a 535 bp segment of the 16s rRNA gene and an 838 bp segment of the COI gene. The COI segment was amplified using primers COIf 5′-CCTGCAGGAGGAGGAGAYCC-3′ (Palumbi and Benzie, 1991) and TL2N 5′-ATGCATATCTATCTGCCATTTT AG-3′ (Quan *et al*., 2001). The 16s rRNA gene was amplified using primers L2510 5′-CGCCTGTTTAACAAAAACAT-3′ and H3059 5′-CCGGTCTAGACTCA GATCATGT-3′ (Bouchon *et al*., 1994).

PCR consisted of 35 cycles with 50 s at 95°C, 1 min at 58°C (1 min at 52°C for 16s rRNA), 1 min at 72°C, and a final 10 min extension at 72°C, with an initial denaturation step at 95°C for 3 min.

Sequencing

The PCR products were purified and sequenced with an FS kit from ABI (Zhang *et al*., 1999). Both strands were sequenced using the primers for PCR.

Sequence Comparison and Phylogenetic Analysis

The sequences were edited and aligned by Editseq and Megalign using the DNAstar software. Sequence variations of COI and 16s rRNA genes were analyzed using MEGA2.0. The distance matrix was computed using Kimura's two-parameter method.

Homologous sequences from three other Crustacean species [*Portunus trituberculatu* (in this paper), *Petrolithes cinctipes* and *Cancer oregonensis* (Harrison and Crespi, 1999)] were used as outgroups. Combined sequences of COI and 16s rRNA were used to carry out the phylogenetic analysis using PAUP 4.0. Three analysis methods (neighbor-joining method, maximum parsimony method, maximum-likelihood method) were employed to verify whether alternative topologies were supported by different tree-building methods. In all cases, the robustness of the trees obtained was determined by the bootstrap resampling procedure (1000 replicates for NJ and MP method, 50 replicates for ML method, heuristic option).

RESULTS

Base Composition of the Two Genes

Sequences of the 847 bp section of the COI gene and the 514 bp section of the 16s rRNA gene were obtained from 42 specimens of these 13 species. Twenty-one COI gene haplotypes and thirteen 16s rRNA gene haplotypes were obtained. The nucleotide sequences of different haplotypes are available in GeneBank under the accession numbers AY264886-AY264903 and AY264904-AY264916.[7] The base composition differs slightly among species (Table II). The mean AT content is 63.3% in the COI gene and 66.25% in the 16s rRNA gene. The rarest base is G (average 16.2%) in the COI gene and C (average 12.63%) in the 16s rRNA gene. These patterns of base composition are consistent with the descriptions of other arthropod mtDNA sequences (Crozier *et al*., 1989; Garcia-Machado *et al*., 1993; Howland and Hewitt, 1995; Navajas *et al*., 1998, 1999; Satta and Takahata, 1990; Spicer, 1995) as well as other marine crustacean mtDNA sequences (Baldwin *et al*., 1998; Harrison and Crespi, 1999; Meyran *et al*., 1997; Palmero *et al*., 1988).

Sequence Variation of the COI Gene

Among the 21 haplotypes, five species, *Penaeus chinensis*, *P. japonicus*, *P. penicillatus*, *Metapenaeus affinis*, and *Metapenaeus ensis,* are polymorphic. Four haplotypes were obtained from 24 samples of *Penaeus chinensis*, and four haplotypes from four samples of *Metapenaeus affinis*. *Penaeus japonicus*, *Penaeus penicillatus*, and *Metapenaeus ensis* each produced two haplotypes.

Sequence alignment of 21 haplotypes of 12 taxa together with the outgroup sequences from *Portunus trituberculatus* showed that 343 of the 847 nucleotide sites (40.50%) were variable without any insertions or deletions. The 294 (85.71%) variable sites were parsimony informative polymorphic sites. Most variations (261 sites) occurred at the third codon position. While 65 variable sites were in the first position, only 17 were in the second position. Most of the mutation events were transitions (56.64%, including 74.07% TC and 25.93% AG). Transversions were mostly AT (65%) and AC (23.3%), with TG (8.3%) and CG (3.3%) occurring much less frequently. On average, the Ti/Tv ratio among the 22 haplotypes was 1.31.

Amino acid sequences of the 847 bp COI fragment were deduced based on the genetic code of *Drosophila* mtDNA. They revealed 282 residues in length, including 51 (18.1%) variable sites. Nearly half (27, 9.6%) of these variable sites occurred among ingroups; the remaining variable sites were between ingroups and outgroup.

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[7] Sequences in this report have been deposited in the GeneBank database as Accession Nos. AY264904-AY264916, and AY264886-AY264903.

Table II. Base Composition (%) of COI Gene and 16s rRNA Gene Among 13 Species Together With the Outgroup *Portunus trituberculatus*

	COI					16s rRNA				
	A%	C%	G%	T%	Sites	A%	C%	G%	T%	Sites
(1) Maff	28.5–28.7	22.2–22.3	17.0–17.1	32.1–32.2	847	28.7	12.7	22.8	35.8	505
(2) Mens	28.7	22.7–22.8	16.2	32.3–32.5	847	29.4	12.9	22.5	35.2	503
(3) Mbar	28.8	18.5	15.2	37.4	847	31.6	12.7	21.5	34.2	503
(4) Pcan	27.3	19.7	16.9	36.1	847	31.8	13.1	21.3	33.8	503
(5) Pchi	28.1–28.3	18.8–18.9	16.2–16.3	36.6–36.7	847	33.1	13.1	20.4	33.5	505
(6) Pjap	27.0–27.5	19.8–20.1	16.3–16.9	36.1–36.4	847	32.0	13.3	21.1	33.6	503
(7) Ppen	27.3	19.8–20.1	16.6	36.0–36.2	847	33.3	12.9	20.2	33.5	504
(8) Pfis	29.2	21.8	15.0	34.0	847	31.0	12.1	21.3	35.6	506
(9) Phar	29.3	19.2	15.1	36.4	847	31.1	11.6	21.8	35.5	501
(10) Pvan	27.0	19.7	16.2	37.1	847	31.9	13.9	21.6	32.5	504
(11) Scra	28.9	22.8	14.8	33.5	847	31.4	12.5	22.1	34.0	506
(12)Tcur	27.6	19.6	16.3	36.5	847	32.7	11.9	20.0	35.5	505
(13)Ptris	26.9	20.3	14.5	38.3	847	35.4	11.6	18.1	34.8	508
Mean	28.1	20.5	16.2	35.2	847	31.8	12.6	21.1	34.4	504.3

Pairwise distances of the COI sequence among the 12 shrimp species and the outgroup *Portunus trituberculatus* are summarized in Table III. The sequence divergence ranged from 0 to 1.20% (within Ppen) within species, 6.5% (Pcan & Pjap) to 21.09% (Pcan & Ppen) within genera, 18.67% (Pfis & Mbar) to 25.39% (Phar & Ppen) between genera, 18.70% (Scar & Pfis) to 22.88% (Scar & Ppen) between families, and 26.38% (Ptris & Scar) to 31.87% (Ptris & Mens) between suborders.

Sequence Variation of 16s rRNA Gene

Twenty-one insertions/deletions and 205 variable sites were observed in the 514 bp section of the 16s rRNA gene of the 12 shrimp species and the outgroup *P. trituberculatus*. Of the variable sites, 117 were parsimony-informative polymorphic sites. No intraspecific variation was found among the 41 samples of 13 taxa. Mean Ti/Tv ratio among the 13 taxa was 1.28.

Table IV shows the pairwise distance of the 16s rRNA gene among the 12 shrimp species and the outgroup *Portunus trituberculatus*. The sequence divergence ranged from 1.21% (between Pjap and Pcan) to 10.73% (between Pchi and Pvan) within genera, 5.81% (Pfis and Mbra) to 18.23% (Phar and Pvan) between genera, 9.40% (Scra and Pfis) to 15.87% (Scra and Pvan) between families, and 26.02% (Ptris and Tcur) to 32.33% (Ptris and Maff) between suborders.

Phylogenetic Analysis

Phylogenetic analysis was carried out based on the 1361 bp combined sequences of COI and 16s rRNA, using three tree-building methods (maximum parsimony, neighbor-joining, and maximum likelihood). The topologies of the three phylogenetic trees are identical except for three weaker bootstrap values in the ML tree (Fig. 1). The 21 haplotypes are clustered into three obvious clades with support of high bootstrap values in all three trees. All the 10 haplotypes of five *Penaeus* species cluster to the first clade. In this clade, *P. canaliculatus* (Pcan) and *P. japonicus* (Pjap) form a sister group while *P. chinensis* (Pchi), *P. penicillatus* (Ppen), and *P. vannamei* (Pvan) form another group. *Metapenaeopsis barbata* (Mbar) gather with *Parapenaeus fissuroides* (Pfis) first before gathering with *Sloenocera crassicornis* (Scra), forming the second clade. *Metapenaeus affinis* (Maff) and *M. ensis* (Mens) cluster first before gathering with the group of *Parapenaeopsis hardiwickii* (Phar) and *Trachypenaeus curvirostris* (Tcur), forming the third clade.

DISCUSSION

Use of the Combined Sequence Data

In this study, two separate gene fragments were sequenced, but the combined sequence data of both COI and 16s rRNA were used for the final phylogenetic

Table III. Pairwise Genetic Distance Among 12 Shrimp Species, Together With the Outgroup *Portunus trituberculatus*, Revealed from COI Nucleotide and Amino Acid Sequences

	1	2	3	4	5	6	7	8	9	10	11	12	13
(1) Maff	0.0047												
(2) Mens	0.1534	0.0036											
(3) Mbar	0.1938	0.21329											
(4) Pcan	0.2119	0.2394	0.1958										
(5) Pchi	0.2160	0.2129	0.206	0.1796	0.00237								
(6) Pjap	0.1914	0.2113	0.1880	0.0650	0.1711	0.0083							
(7) Ppen	0.2067	0.2131	0.2170	0.2109	0.1498	0.1903	0.0120						
(8) Pvan	0.2089	0.2046	0.1912	0.1896	0.1761	0.1968	0.1765						
(9) Pfis	0.2004	0.2209	0.1867	0.2159	0.2110	0.2113	0.2304	0.2095					
(10) Phar	0.2047	0.2192	0.1939	0.2071	0.2215	0.2003	0.2539	0.2314	0.1913				
(11) Scra	0.2033	0.1956	0.2023	0.1994	0.2000	0.20112	0.2288	0.2099	0.1870	0.2120			
(12) Tcur	0.2240	0.2111	0.2135	0.2328	0.2533	0.2418	0.2481	0.2343	0.2237	0.1875	0.2257		
(13) Ptris	0.2911	0.3187	0.2709	0.2697	0.2916	0.2805	0.3020	0.2651	0.2945	0.2791	0.2638	0.2878	

Table IV. Pairwise Genetic Distance Between 12 Shrimp Species, Together With the Outgroup *Portunus trituberculatus*, Revealed From 16s rRNA Nucleotide Sequence

	1	2	3	4	5	6	7	8	9	10	11	12	13
(1) Maff													
(2) Mens	0.0495												
(3) Mbar	0.1215	0.1315											
(4) Pcan	0.1633	0.1610	0.1495										
(5) Pchi	0.1751	0.1706	0.1438	0.0889									
(6) Pjap	0.1659	0.1637	0.1573	0.0121	0.0889								
(7) Ppen	0.1701	0.1681	0.1414	0.0846	0.0349	0.0869							
(8) Pvan	0.1710	0.1766	0.1508	0.1047	0.1073	0.1118	0.0959						
(9) Pfis	0.1086	0.1114	0.0581	0.1270	0.1313	0.1246	0.1266	0.1384					
(10) Phar	0.1312	0.1269	0.1446	0.1517	0.1512	0.1569	0.1463	0.1823	0.1151				
(11) Scra	0.1528	0.1504	0.1278	0.1396	0.1341	0.1371	0.1344	0.1587	0.0940	0.1322			
(12) Tcur	0.1430	0.1360	0.1345	0.1290	0.1453	0.1291	0.1356	0.1557	0.0936	0.0949	0.1438		
(13) Ptris	0.3233	0.3204	0.3063	0.2819	0.2865	0.2727	0.2720	0.2982	0.2769	0.2905	0.3193	0.2602	

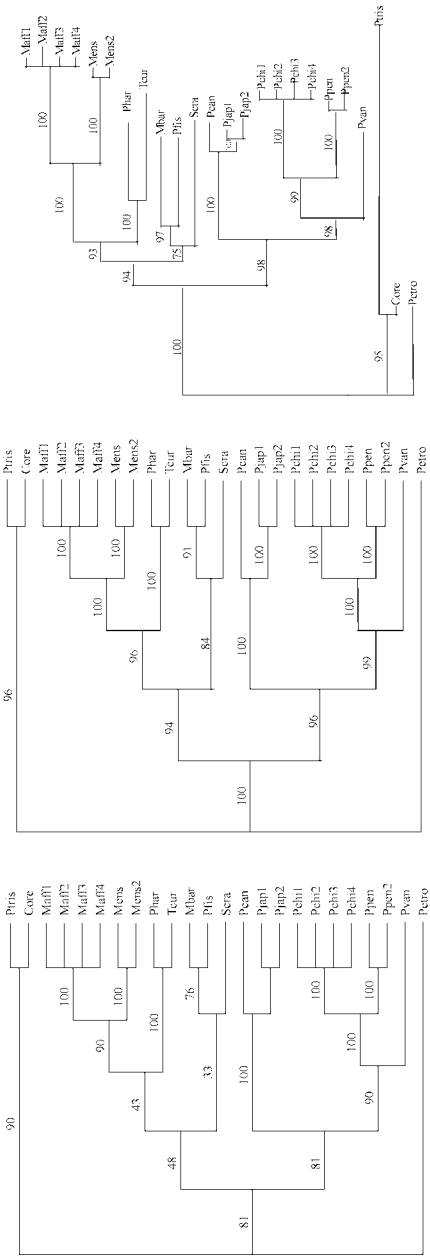

Fig. 1. Phylogenetic trees of 21 haplotypes together with outgroup sequences from *Portunus trituberculaus* (Ptris), *Petrolithes cinctipes* (Petro: Harrison and Crespi, 1999) and *Cancer oregonensis* (Core: Harrison and Crespi, 1999). (a) Maximum likelihood (ML) tree with bootstrap values for 50 replicates above each branch; (b) maximum parsimony (MP) tree with bootstrap values for 1000 replicates above each branch (length = 1061, CI = 0.7531, RI = 0.8396, RC = 0.6322); (c) neighbor joining (NJ) tree with bootstrap values for 1000 replicates above each branch (branch lengths are proportional to divergence).

analysis. Such assay could be explained by three main reasons. First, our phylogenetic analysis based only on the 847 bp COI sequences could solve the relationships of the closely related taxa with support of high bootstrap values but could not get the consensus relationships of *Metapenaeopsis barbata* (Mbar), *Parapenaeus fissuroides* (Pfis), and *Solenocera crassicornis* (Scra) via three different methods. Second, our phylogenetic analysis based merely on the 514 bp 16s rRNA gene sequences could not solve the relationships of the four *Penaeus* species through the three methods, although the relationships of other taxa were well solved. Finally, phylogenetic analysis based on combined data of different independent sites normally is helpful to get more exact results (Kluge, 1989; Zhang, 1996; Zhang and Ryder, 1994). In this study, therefore, using the 1361 bp combined sequences of both the COI gene and 16s rRNA gene, three different analytical methods produced an identical topology with support from high bootstrap confidence. As a result, the reliable phylogenetic relationship of these 12 Penaeoidea shrimp species was well authenticated at the molecular level.

Intraspecific Variation

Although this study scanned a limited number of taxa, intraspecific variation ranging from 0.24 to 1.20% (Table III) in the COI gene fragment was revealed, while no intraspecific variation was detected in the 16s rRNA gene fragment. These results indicate that the fragment of the COI gene chosen here appears to be a good marker for speciation studies and population analysis in Crustaceans, while the 16s rRNA gene fragment seems suitable for examining the phylogenetic relationships at the species or higher taxonomic levels in Crustacean. This suggestion is concordant with the results of some similar studies on other arthropods (Baldwin *et al.* 1998; Meyran *et al*., 1997; Navajas *et al*., 1998, 1999; Quan *et al*., 2001). For instance, 5% intraspecific variation was found in the COI gene sequence of *Tetranychus urticae* (Navajas *et al*., 1998). Based on a sequence analysis of the COI gene, the sequence divergence across different haplotypes of *Ampnitetranychus viennen* ranged from 3.8 to 4.1% (Navajas *et al*., 1999). Up to 8.2% intraspecific variation was found in the COI sequence of *Grammarus pulex* (Meyran *et al*., 1997). About 0–3 % intraspecific variation was observed in a sequence analysis of the COI gene in 13 *Penaeus* species (Baldwin *et al*., 1998). The 16s rRNA sequences were successfully used to analyze the systematic relationship and phylogeographic history of 46 Eastern Pacific porcelain crab species (Stillman and Reeb, 2001). The 16s rRNA gene was also used to investigate molecular phylogeny, taxonomy, and evolution of American grapsoid crabs (Schubart *et al*., 2000). The 1416 bp nucleotide sequences of mitochondrial 12s rRNA to 16s rRNA genes of 20 crab species, representing four recognized subfamilies of Ocypodidae, were also used to analyze phylogeny and evolution of a mud-using behavior in ocypodid crabs (Kitaura *et al*., 1998).

Our limited sequence data (Table III) show that the lowest interspecific variation was several times higher than the highest intraspecific variation in Penaeidae, which suggests that the intraspecific variation would have little impact on the phylogeny in Penaeidae.

Phylogenetic Relationship

As for the divergences of the various taxa in Penaeidae, there are two different opinions. Based on morphological features, Burkenroad (1983) divided the Penaeidae into three tribes: the Peneini (*Penaeus*, *Heteropenaers*, *Funchalia*, *Pelagopenaeus*), the Parapeneini (*Parapenaeus, Artemesia, Penaeopsis, Metapenaeopsis*), and the Trachypeneini (*Metapenaeus, Macropetasma, Trachypenaeopsis, Atypopenaeus Protrachypene, Xiphopenaeus, Parapenaeopsis, Trachypenaeus*). While using similarity matrices computed on the basis of a complex classification of morphological features, Kubo (1949) distinguished Penaeidae as five groups: group 1 (*Penaeus*), group 2 (*Penaeopsis*), group 3 (*Atypopenaeus, Trachypenaeopsis, Metapenaeus*), group 4 (*Parapenaeus, Parapenaeopsis, Trachypenaeus*), and group 5 (*Metapenaeopsis*). Group 4 diverged a little earlier than the other four groups that diverged together. According to our results, phylogenetic analyses in the present paper indicate that species-group assignment based on the 1361 bp combined sequences of COI and 16s rRNA (Fig. 1) is obviously consistent with the opinion of Burkenroad (1983). Namely, five species of *Penaeus* (Pcan, Pjap, Pchi, Ppen, Pvan) belong to the tribe Peneini, *Parapenaeus* (Pfis) and *Metapenaeopsis* (Mbar) pertain to the tribe Parapeneini, while *Metapenaeus* (Maff, Mens), *Parapenaeopsis* (Phar), and *Trachypenaeus* (Tcur) belong to the tribe Trachypeneini.

According to the traditional taxonomy, Penaeoidea can be divided into four families: Aristaeidae, Solenoceridae, Penaeidae, and Sicyonidae. *Solenocera crassicorni* belongs to the family Solenoceridae (Dall *et al*., 1990). Based on the results of the present study, however, it seems more reasonable to class *Solenocera crassicorni* in the tribe Parapeneini in the family Penaeidae. There are two explanations for this suggestion. First, the largest genetic distance between Scar and other taxa in Penaeidae (15.87% in the 16s rRNA gene, 22.88% in the COI gene) is much smaller than the largest distance between genera in Penaeidae (18.23% in 16s rRNA gene, 25.39% in COI gene, in Tables III and IV). Second, the positions of *Sloenocera crassicornis* (Scar) are identical in all three phylogenetic trees (Fig. 1), namely, it is the sister clade of the group of *Metapenaeopsis barbata* (Mbar) and *Parapenaeus fissuroides* (Pfis).

The divergence rate of the COI gene in *Penaeus* was estimated to be approximately 3% per million years (Baldwin *et al*., 1998). In the family Penaeidae, the ancient Mesozoic genus *Penaeus* was in existence before the tropical Atlantic was closed off, and it is the only genus that has survived through the Cainozoic

Era (Dall *et al*., 1990). Therefore, we can use this rate to estimate the separation time of some taxa in the family Penaeidae. Mulley and Latte (1980) indicated that the *Penaeus* and *Metapenaeus* separated about 4.7×10^6 years ago in the late Pliocene, and our results suggest that the two genera separated about post-Miocene. Because 19.14–23.94% sequence divergence in the COI sequence was observed between *Penaeus* and *Metapenaeus* (Table III), the approximate separation time is estimated to be about 6.38×10^6–7.98×10^6 years, suggesting that *Penaeus* and *Metapenaeus* might have separated about 6.38×10^6–7.98×10^6 years BP in the post-Miocene. Interspecific divergence of the COI sequence within *Penaeus* and *Metapenaeus* ranges from 6.5 to 7.03% (Table III), giving an estimate of species separation ranging from 2.16×10^6 to 7.03×10^6 years BP in the Pliocene, which is within the separation period indicated by Mulley and Latte (1981), from the middle of the Tertiary to the late Pleistocene. Intraspecific sequence divergence within two *Metapenaeus* species and three *Penaeus* species ranges from 0.24 to 1.2% (Table III), suggesting the separation of these five species might have occurred 0.08×10^6–0.4×10^6 years BP in the late Pleistocene. This suggestion supports the opinion that the present penaeid species could have arisen during the last glacial epoch (Dall *et al*., 1990). However, the number of the tested taxa in this study is limited. Further studies on more taxa in this family are desirable to further understand the phylogeny of the superfamily Penaeoidae.

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