



Evaluation of a novel 16S rRNA/tRNA^{Val} mitochondrial marker for the identification and phylogenetic analysis of shrimp species belonging to the superfamily Penaeoidea

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ARTICLE INFO

Article history:

Received 28 March 2009

Available online 18 May 2009

Keywords:

Mitochondrial DNA

Penaeid shrimp

Decapoda

16S rRNA

Cytochrome c oxidase I (COI)

Species identification

Food authenticity

PCR-RFLP

Phylogenetic analysis

ABSTRACT

In this study, we infer the phylogenetic relationships within commercial shrimp using sequence data from a novel mitochondrial marker consisting of an approximately 530-bp region of the 16S ribosomal RNA (rRNA)/transfer RNA (tRNA)^{Val} genes compared with two other mitochondrial genes: 16S rRNA and cytochrome c oxidase I (COI). All three mitochondrial markers were considerably AT rich, exhibiting values up to 78.2% for the species *Penaeus monodon* in the 16S rRNA/tRNA^{Val} genes, notably higher than the average among other Malacostracan mitochondrial genomes. Unlike the 16S rRNA and COI genes, the 16S rRNA/tRNA^{Val} marker evidenced that *Parapenaeus* is more closely related to *Metapenaeus* than to *Solenocera*, a result that seems to be more in agreement with the taxonomic status of these genera. To our knowledge, our study using the 16S rRNA/tRNA^{Val} gene as a marker for phylogenetic analysis offers the first genetic evidence to confirm that *Pleoticus muelleri* and *Solenocera agassizi* constitute a separate group and that they are more related to each other than to genera belonging to the family Penaeidae. The 16S rRNA/tRNA^{Val} region was also found to contain more variable sites (56%) than the other two regions studied (33.4% for the 16S rRNA region and 42.7% for the COI region). The presence of more variable sites in the 16S rRNA/tRNA^{Val} marker allowed the interspecific differentiation of all 19 species examined. This is especially useful at the commercial level for the identification of a large number of shrimp species, particularly when the lack of morphological characteristics prevents their differentiation.

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Decapoda marine shrimp, especially those belonging to the superfamily Penaeoidea, represent the majority of the world's commercially important shrimp species, accounting for more than 80% of the wild catch [1]. Among them, shrimp of the families Penaeidae and Solenoceridae are valuable resources for fisheries and aquaculture in both tropical and subtropical regions. The identification and characterization of shrimp traditionally relied on morphometric analysis; however, it is well known that such characteristics are environmentally influenced [2]. Accordingly, genetic studies of shrimp populations have been conducted using a variety of approaches such as isozyme protein electrophoresis [3], microsatellite analysis [4,5], and allozyme analysis [6,7]. Nuclear genes such as the 28S ribosomal RNA (rRNA),¹ 18S rRNA, 5.8S rRNA, and

internal transcribed spacer 1 (ITS1) have also been considered for the study of phylogenetic relationships among shrimp [8–10]. Nevertheless, the unique properties of mitochondrial DNA (mtDNA), especially the fact that its evolutionary rate is 10 times faster than that of nuclear DNA, make it a particularly useful tool in phylogenetic analyses. The most commonly used molecular markers include the 16S rRNA, 12S rRNA, and cytochrome c oxidase subunit I (COI) genes, the mtDNA control region, and (more recently) the cytochrome *b* gene (*cytb*) [1,6,11–15]. Such mtDNA markers have been used not only for the phylogenetic classification of natural shrimp populations [1,5,6,16] but also for the elucidation of genetic divergence between captive populations [17,18].

Among the mtDNA markers, the 16S rRNA and COI have been previously used for inferring phylogenetic relationships among shrimp species. On the one hand, studies indicate that the 16S rRNA region exhibits a low rate of evolution [19], meaning that it is especially useful for the interspecific differentiation of species rather than for the intraspecific differentiation of specimens. Accordingly, certain studies based on the 16S rRNA gene have established evolutionary relationships among shrimp at different

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¹ Abbreviations used: rRNA, ribosomal RNA; ITS1, internal transcribed spacer 1; mtDNA, mitochondrial DNA; COI, cytochrome c oxidase subunit I; *cytb*, cytochrome *b*; tRNA, transfer RNA; PCR, polymerase chain reaction; SNP, single nucleotide polymorphism; RFLP, restriction fragment length polymorphism; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; NJ, neighbor-joining.

taxonomic levels [20–23]. On the other hand, COI has proven to be a robust evolutionary marker for determining both intraspecific and interspecific relationships in a variety of marine mollusks [1,24]. In the same vein, a previous report showed high genetic variation in the mitochondrial COI gene within some shrimp species [24]. Thus, the genetic divergences observed in the COI gene among different shrimp species are consistently higher than those reported for the 16S rRNA gene, with the former exhibiting sufficient nucleotide diversity to allow the detection of different haplotypes [25].

In this study, we investigated a novel mitochondrial marker in the 16S rRNA/transfer RNA (tRNA)^{Val} region and compared it with the COI and 16S rRNA genes in an effort to elucidate which of these markers constitutes a better molecular tool both for the basic investigation of the phylogenetic relationships among shrimp species and to assess the authenticity and traceability of most commercially relevant species.

Materials and methods

Shrimp species examined

In this study, we compared a 530-bp fragment of the 16S rRNA/tRNA^{Val} region with nucleotide sequences of two mitochondrial genes, namely a 449-bp fragment of the COI and 476 bp of the 16S rRNA, both of which were reported previously by others and, therefore, were available for download from the GenBank database. The shrimp species that we evaluated account for the most marketed species.

The 24 species of shrimp (superfamily Penaeoidea) included in this study belong to two families: Penaeidae and Solenoceridae. Their scientific names, commercial names, and accession numbers are compiled in Table 1. These included genera belonging to the family Penaeidae, such as *Farfantepenaeus*, *Fenneropenaeus*, *Litopenaeus*, *Penaeus*, *Marsupenaeus*, *Melicertus*, *Metapenaeus*, and

Parapenaeus, as well as the family Solenoceridae, represented by the genera *Solenocera* and *Pleoticus*.

DNA extraction, amplification, sequencing, and restriction fragment length polymorphism analysis

Extraction and amplification protocols were as described elsewhere [14]. Briefly, samples of 250 mg of skeletal muscle from each shrimp specimen were obtained. In all cases, three different specimens were considered for each species. DNA was extracted by means of a commercial kit (DNeasy Tissue Kit, Qiagen, Darmstadt, Germany), as described previously [14]. The primers used for the polymerase chain reaction (PCR) amplification and sequencing of the 16S rRNA/tRNA^{Val} genes were 16ScruC4 (5'-AATATGGCTG TTTTAAAGCCTAATCA-3') and 16ScruC3 (5'-CGTTGAGAAGTTCG TTGTGCA-3'), which were constructed on two well-conserved regions of the 16S rRNA/tRNA^{Val} genes. Such primers allowed the amplification of a 515- to 535-bp fragment of the 16S rRNA/tRNA^{Val} mtDNA genes in the penaeid shrimp species considered, as described elsewhere [14].

Sequencing was performed in both directions. Prior to sequencing, the PCR products were purified by means of the ExoSAP-IT kit (GE Healthcare, Uppsala, Sweden). Direct sequencing was performed with the BigDye Terminator Cycle Sequencing Kit (version 3.1, Applied Biosystems, Foster City, CA, USA). The same primers used for PCR were also employed for the sequencing of both strands of the PCR products. Sequencing reactions were analyzed in an automatic sequencing system (ABI 3730XL DNA Analyzer, Applied Biosystems) provided with the POP-7 system. Single nucleotide polymorphism (SNP) events in DNA sequences were carefully reviewed by eye using Chromas software (Griffith University, Queensland, Australia). Sequence alignment was accomplished using ClustalX 1.8 software [26]. Sequence homologies were searched using the BLAST tool (National Center for Biotechnology Information).

Table 1
Shrimp species considered in this study, common names, 3-alpha codes, 16S rRNA/tRNA^{Val}, 16S rRNA, and COI sequence GenBank accession numbers.

Taxonomic designation	Common name	FAO species code	16S rRNA/ tRNA ^{Val} Accession Nos.	16S rRNA Accession Nos.	COI Accession Nos.
Family Penaeidae (penaeid shrimp)					
<i>Farfantepenaeus aztecus</i>	Northern brown shrimp	ABS	EF589699 ^a	AF192051	AF279834
<i>Farfantepenaeus brasiliensis</i>	Redspotted shrimp	PNB	EF589701 ^a	AF192054	AF029393
<i>Farfantepenaeus brevisrostris</i>	Crystal shrimp	CSP	EF589700 ^a	NA	NA
<i>Farfantepenaeus californiensis</i>	Yellowleg shrimp	YPS	AY046912	AY046912	AY135197
<i>Farfantepenaeus notialis</i>	Southern pink shrimp	SOP	EF589694 ^a	NA	X84350
<i>Fenneropenaeus chinensis</i>	Fleshy prawn	FLP	NC_009679	AF245113	DQ778650
<i>Fenneropenaeus indicus</i>	Indian white prawn	PNI	EF589688 ^a	AF279815	AF279837
<i>Fenneropenaeus merguensis</i>	Banana prawn	PBA	EF589692 ^a	AF279814	AY143990
<i>Litopenaeus setiferus</i>	Northern white shrimp	PST	AJ297971	AJ297971	NA
<i>Litopenaeus stylirostris</i>	Blue shrimp	PNS	AY046913	AF255054	AY135191
<i>Litopenaeus vannamei</i>	Whiteleg shrimp	PNV	EF589702 ^a	AY344189	NC_009626
<i>Penaeus monodon</i>	Giant tiger prawn	GIT	AF217843	NC_002184	NC_002184
<i>Penaeus semisulcatus</i>	Green tiger prawn	TIP	EF589704 ^a	AY744268	AF279831
<i>Marsupenaeus japonicus</i>	Kuruma prawn	KUP	NC_007010	AY742276	NC_007010
<i>Melicertus latisulcatus</i>	Western king prawn	WKP	EF589708 ^a	AF279821	AF279845
<i>Metapenaeus ensis</i>	Greasyback shrimp	MPE	NA	AF279810	AF279830
<i>Metapenaeus affinis</i>	Jinga shrimp	MTJ	NA	AY264904	AY264889
<i>Metapenaeus sp</i>	Metapenaeus shrimp nei	MET	EF589713 ^a	NA	NA
<i>Parapenaeus longirostris</i>	Deep-water rose shrimp	DPS	EF589715 ^a	NA	NA
<i>Parapenaeus fissuroides</i>	-	PAF	NA	AY264909	AY264894
Family Solenoceridae (solenocerid shrimp)					
<i>Pleoticus muelleri</i>	Argentine red shrimp	LAA	EF589716 ^a	NA	NA
<i>Solenocera agassizi</i>	Kolibri shrimp	SOK	EF589719 ^a	NA	NA
<i>Solenocera crassicornis</i>	Coastal mud shrimp	SOJ	NA	AY264915	AY264902
<i>Solenocera koelbeli</i>	Chinese mud shrimp	SKO	NA	AF105038	AF105049
Family Palinuridae (spiny lobsters)					
<i>Panulirus japonicus</i>	Japanese spiny lobster	NUJ	NC_004251	NC_004251	NC_004251

Note: NA, not available.
^a This study.

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