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Phylogenetic relationships among genera of the *Periclimenes* complex (Crustacea: Decapoda: Pontoniinae) based on mitochondrial and nuclear DNA

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ABSTRACT

The genus *Periclimenes* Costa, 1844 is the most species-rich genus in the subfamily Pontoniinae. Recent studies have suggested that it might be a polyphyletic taxon and could be further subdivided. In this study, three protein-coding nuclear genes and one mitochondrial ribosomal gene were used to analyze the phylogenetic relationships among the genera of the *Periclimenes* complex, includes the genus *Periclimenes* and 15 related genera, viz. *Ancylomenes*, *Brucecaris*, *Crinotonia*, *Cuapetes*, *Harpiliopsis*, *Harpilius*, *Laomenes*, *Leptomenaeus*, *Manipontonia*, *Palaemonella*, *Periclimenella*, *Philarius*, *Phycomenes*, *Unguicaris* and *Vir*. Based on both independent and combined data analyses, the results support that the genus *Periclimenes* is a polyphyletic group. Furthermore, the studied *Periclimenes* species could be divided into several independent groups, and the taxonomic status of *P. commensalis*, *P. brevicarpalis* and *P. digitalis* may need to be reconsidered. Besides, the majority of the related genera of *Periclimenes* are suggested to be monophyletic. Our analyses also reveal that these genera approximately form two main clades, despite some deep relationships are still obscure.

1. Introduction

Caridean shrimps are one of the most diverse groups of Decapoda. Since it was erected, many classification schemes of Caridea were proposed, but the current classification of Caridea is still unnatural (De Grave and Fransen, 2011). There are many controversial phylogenetic issues remained to be resolved, and these issues include the relationships among genera of the *Periclimenes* complex. To date, the genus *Periclimenes* Costa, 1844 includes more than 150 species (De Grave and Fransen, 2011; De Grave et al., 2009), and is the most species-rich genus in the subfamily Pontoniinae (Li, 2009) of the caridean shrimp. Members of the genus are widely distributed in all oceans (Bruce, 2007) and occur from the intertidal zone to the deep sea. Many of them are free-living, but some species commensally associate with various marine invertebrates, including sponges, cnidarians, mollusks and echinoderms (Bruce, 2007). Their high diversity in morphology and life style indicate that the genus *Periclimenes* is not a monophyletic group (Bruce, 2007; Li, 2009).

Since Costa (1844) established the genus *Periclimenes*, its taxonomy has been revised mainly based on the morphological characters. Recently, a few genera synonymous with *Periclimenes* were resurrected (Bruce, 2007; Li, 2009). *Hamiger* Borradaile, 1916 is the first genus resurrected by Bruce (1986), with a single species *Hamiger novaezealandiae* Borradaile, 1916. Holthuis (1952) reduced *Harpilius* Dana, 1852 to a subgenus of *Periclimens* Costa, but Bruce (2004) resurrected it to a separate genus, which consists of four species. The genus *Kemponia* was also erected by Bruce (2004). Subsequently, Okuno (2009) resurrected the genus *Cuapetes* Clark, 1919, as a senior synonym of *Kemponia* Bruce, 2004, and now *Cuapetes* includes 26 species (Bruce, 2012; Okuno and Chan, 2012). Okuno and Fujita (2007) reestablished the genus *Laomenes* Clark, 1919 with three species at first. After that, six more species were transferred or added into this genus (Li, 2009; Marin, 2009; Marin and Okuno, 2010; Marin et al., 2012). Moreover, some species were separated from *Periclimenes* and erected as new genera (Bruce, 2007; Li, 2009). *Zenopontonia* Bruce, 1975, *Exoclimenella* Bruce, 1995, *Periclimenella* Bruce, 1995, *Manipontonia* Bruce, Okuno and Li, 2005, *Leptomenes* Bruce, 2006 (renamed as *Leptomenaeus* Bruce, 2007), *Crinotonia* Marin, 2006, *Brucecaris* Marin and Chan, 2006, *Unguicaris* Marin and Chan, 2006, *Rapipontonia* Marin, 2007, *Margitonia* Bruce, 2007, *Pseudoclimenes* Bruce, 2008, *Phycomenes* Bruce, 2008, *Sandimenes* Li, 2009 and *Ancylomenes* Okuno and Bruce, 2010 were established in succession. Furthermore, the restricted genus *Periclimenes* were divided into many species groups, such as the *obscurus* group (Bruce, 1987), *diversipes* group (Bruce, 1989), *foresti* group (Bruce, 1981;

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Li et al., 2008), *alcocki* group (Li and Bruce, 2006; Li et al., 2008) and *brevicarpalis* group (Bruce, 2010b). These widely accepted species groups suggest the genus *Periclimenes* probably is a polyphyletic taxon and could be further subdivided (Bruce, 2007).

Definitely, with further research, more genera will be separated from *Periclimenes* and the genus will be defined more accurately. However, so far the taxonomic and systematic researches of *Periclimenes* and related genera are mainly based on morphology. Even in the superfamily Palaemonoidea, except of the genus *Macrobrachium* Bate, 1868, the molecular systematics was poorly studied. Only the systematic positions of several species–poor palaemonoidean families such as Gnathophyllidae, Hymenoceridae (Mitsuhashi et al., 2007) and Kakaducaridiidae (Page et al., 2008) were explored, mainly based on mitochondrial (COI, 16S) and nuclear (18S, 28S) ribosomal DNA sequences. In recent years nuclear protein-coding genes have been increasingly used to resolve the systematics of decapod. Sodium–potassium ATPase α-subunit (NaK), phosphoenolpyruvate carboxykinase (PEPCK), occasionally coupled with histone 3 genes were utilized to reconstruct the phylogeny of the Reptantia (Tsang et al., 2008), Penaeoidea (Ma et al., 2009) and Palinuridae (Tsang et al., 2009). Most recently, Li et al. (2011) examine the systematic status of caridean families and superfamilies based on five nuclear genes, 18S rRNA, enolase, histone 3, PEPCK and NaK. Furthermore, Tsang et al. (2011) provided some fresh insights in the phylogeny of the Anomura by using five nuclear protein-coding genes, arginine kinase (AK), enolase, glyceraldehyde 3-phosphate dehydrogenase (GAPDH), NaK and PEPCK. Due to the higher resolution of the nuclear protein-coding genes at higher taxonomic levels (Li et al., 2011), the present study utilized three nuclear protein-coding genes, enolase, PEPCK and NaK, in addition to 16S rRNA. We attempted to explore the monophyly of the genus *Periclimenes* and provide insights into the phylogenetic relationships between *Periclimenes* and its related genera.

2. Materials and methods

2.1. Sample collection

In this study, a total of 43 species from 16 genera, plus one outgroup species *Macrobrachium nipponense* (De Haan, 1849) were collected. The 16 genera include the genus *Periclimenes* and 15 related genera, which were selected according to Li (2009). All the specimens were identified before being preserved in 75–100% alcohol. The details of all the specimens are listed in Table 1.

2.2. DNA extraction, PCR amplification and sequencing

The abdominal muscle, pleopod or eggs (5–20 mg) of the specimens were used for DNA extraction using a QIAamp DNA Mini Kit (QIAGEN). The DNA was eluted in 100 μl of sterile distilled H_2O (RNase free), and stored in −20 °C freezer. The extracted DNA was checked by 1.5% agarose gel electrophoresis and ethidium bromide staining.

Partial segments of 16S rRNA (~450 bp) and three nuclear genes enolase (~420 bp), NaK (~650 bp) and PEPCK (~550 bp), were amplified by polymerase chain reaction (PCR). The PCR amplifications were performed in a 40 μl reaction, which contained 33-30 μl sterile distilled H_2O, 4 μl of 10 × PCR buffer (Mg^{2+} plus, Takara), 0.8 μl of dNTP (10 mM each), 0.8 μl of each primer (10 μM), 0.4 μl of Taq polymerase (5 unit/μl, Takara) and 1–4 μl of DNA extract. PCR products were purified using the QIAquick Gel extraction Kit (QIAGEN) and sequenced using forward and reverse primers with ABI 3730xl DNA Analyzer (Applied Biosystems).

For the 16S rRNA segments, PCR reactions were carried out using primers 16S-A/B (Wowor et al., 2009), with the following program: initial denaturation for 10 min at 94 °C, followed by 35–40 cycles of denaturation at 94 °C for 30 s, annealing at 50 °C for 40 s, extension at 72 °C for 1 min, and a final extension at 72 °C for 10 min. For the amplification of the enolase, NaK and PEPCK segments, the primers EA2/ES2 (Tsang et al., 2011), FB/NR and PA3/PR3 (Tsang et al., 2008) were used accordingly, and the thermal cycle used was similar to that above except the annealing temperature was changed to 52 °C, 57 °C and 55 °C accordingly.

2.3. Sequence analysis

The sequence chromatograms were checked using Chromas 2.23 by eye. Forward and reverse sequence fragments were assembled using ContigExpress (a component of Vector NTI Suite 6.0). Then the multiple sequence alignments were conducted using Clustal X 1.8.1 (Thompson et al., 1997) under the default parameters, and adjusted by eye. After that, the gappy columns in the beginning and end of the alignments were removed. For the combined analysis, the pruned alignments were concatenated into a single dataset consisting of four genes end to end.

We first tried to use 16S rRNA to describe an overall relationship for all the taxa, and the combined dataset to explore the deeper relationships at the generic level. Maximum likelihood (ML) and Bayesian inference (BI) analyses were used to construct the phylogenetic trees. Two methods were conducted for each dataset separately. ModelTest version 3.7 (Posada and Crandall, 1998) was used to select the best-fit DNA substitution models for ML and BI analyses, respectively. The ML analysis was carried out using PhyML 3.0 (Guindon et al., 2010) performed on the ATGC bioinformatics platform. The node support was evaluated with 1000 bootstrap replicates (BP). The BI analysis was conducted using BEAST v1.6.1 (Drummond et al., 2012). The combined dataset was analyzed using parameters selected by ModelTest from individual dataset. The Markov chains were run for 10,000,000 generations, with sampling every 1000 generations. The first 10% trees were discarded as burn-in, while the remaining trees were used to construct the 50% majority rule consensus tree and to estimate the posterior probabilities (PP). The phylogenetic parameters (e.g. the means and variances of posterior, prior and likelihood scores) were diagnosed by Tracer v1.5 (Rambaut and Drummond, 2007) to make sure the convergence was reached.

3. Results

The 16S rRNA gene alignment consists of 405 bp including all 44 species. As the nuclear genes are much more difficult to be amplified than 16S rRNA gene, all the three protein-coding nuclear genes segments were successfully amplified from 34 of the 44 species studied. The combined alignment of four genes consists of 1982 bp. The characteristics of the four genes, their base frequencies and the substitution models selected by ModelTest are listed in Table 2.

In the 16S rRNA phylogenetic tree (44 taxa, Fig. 1), the monophyly of *Periclimenes* is clearly rejected. Most of the studied *Periclimenes* species formed two groups (A and B, Fig. 1), except the three species *P. brevicarpalis*, *P. commensalis* and *P. digitalis*. The groups A and B are strongly supported in both BI and ML analyses (PP = 1.00, BP = 87%; PP = 1.00, BP = 100%, respectively). *P. brevicarpalis* is close related to the genus *Ancylomenes* with moderate support in both analyses (PP = 0.85, BP = 47%). *P. commensalis* clusters with the genus *Unguicaris* in both analyses with strong support (PP = 0.99, BP = 90%). *P. digitalis* aligns with *Periclimenella spinifera* (De Man, 1902), but the support is weak (PP = 0.64, BP < 50%). Moreover, most of the *Periclimenes* complex form two major clades (I and II, shown in Fig. 1) with high support value in both analyses