Phylogenetic relationships of five species of Dorippinae
(Crustacea, Decapoda) revealed by 16S rDNA
sequence analysis

FAN Yu\textsuperscript{1,2}, LI Xinzheng\textsuperscript{1*}, SONG Linsheng\textsuperscript{1}, CAI Zhonghua\textsuperscript{1}

1. Institute of Oceanology, Chinese Academy of Sciences, Qingdao 266071, China
2. Graduate School, Chinese Academy of Sciences, Beijing 100039, China

Received 11 January 2004; accepted 20 April 2004

Abstract
A molecular phylogeny is presented for the subfamily Dorippinae (including 9 individuals, representing 5 species and 4 genera), based on the sequence data from 16S rRNA gene. Two-cluster test between lineages in these phylogenetic trees has been performed. On the basis of rate constancy, the rate of nucleotide substitutions of 16S rDNA sequence data is estimated as 0.27% per million years. The analysis strongly supports the recognition of the Dorippinae as a monophyletic subfamily. Phylogenetic tree indicates that the subfamily Dorippinae is divided into two main clades, and genus Dorippe appears basal in the subfamily, diverging from other species 36.6 Ma ago. It is also clear that the Heikea is closely related to the genus Neodorippe. The divergence time between them is 15.8 Ma.

Key words: crab, Dorippinae, Dorippe tenuipes, Heikea japonica, Neodorippe callida, Paradorippe granulata, Paradorippe polita, 16S rDNA, phylogeny, divergence time

1 Introduction

The subfamily Dorippinae, one of the two subfamilies of Dorippidae (Crustacea, Brachyura), is a common crab group in shallow waters, 17 species belonging to 8 genera are known from the Indo–West Pacific (Holthuis and Manning, 1990), and 11 species, 7 genera have been recorded from the China’s seas (Chen and Sun, 2002), i.e., Dorippe, with 3 species, Paradorippe and Heikea, containing two species respectively, and Dorippoides, Nobilum, Neodorippe, and Philippidorippe, each containing 1 species.

Recently, the molecular systematics has been used to analyze the phylogenetic relationships of groups or species populations of Crustacea (Liu et al., 2000; Liu et al., 2003) and other marine biological groups (Quan et al., 2000) in China.

In this study, phylogenetic analysis of a collection of species in 4 genera and 5 species is performed using DNA sequence data from a portion of the mitochondrial gene for the ribosomal large subunit (16S rRNA). 16S rDNA sequence analysis has been successfully used...
to construct phylogenies in crabs (e.g., Cunningham et al., 1992; Levinton et al., 1996; Schubart et al., 2000; Schubart et al., 2001). The main objectives of this study are (1) to reconstruct the phylogenetic relationships of these species, (2) to examine the correlations of morphology-based and molecular-based divisions, and (3) to estimate rates of nucleotide substitution for geminate species and calibrate a molecular clock for Dorippinae, thereby enabling the placement of divergence times for all clades.

### 2 Materials and methods

#### 2.1 Sample collection

Crab samples for this study are all taken from the Marine Biological Museum of Chinese Academy of Sciences (MBMCAS) in the Institute of Oceanology, Chinese Academy of Sciences (IOCAS). All material is preserved in 75% ethanol. Nine individuals, representing 5 species and 4 genera of Dorippinae are studied. Data for specimens studied are given in Table 1.

<table>
<thead>
<tr>
<th>Species*</th>
<th>n</th>
<th>Collection locality</th>
<th>CN</th>
<th>GenBank accession No.</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Dorippe</em> Weber, 1795</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Dorippe tenuipes</em> Chen, 1980</td>
<td>2</td>
<td>South China Sea</td>
<td>Q39B–1</td>
<td>AY452771</td>
</tr>
<tr>
<td></td>
<td></td>
<td>South China Sea</td>
<td>K82B–17</td>
<td>AY452772</td>
</tr>
<tr>
<td>Genus <em>Heikea</em> Holthuis and Manning, 1990</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Heikea japonica</em> (von Siebold, 1824)</td>
<td>2</td>
<td>East China Sea</td>
<td>V310B–6</td>
<td>AY452769</td>
</tr>
<tr>
<td></td>
<td></td>
<td>East China Sea</td>
<td>D7B–10</td>
<td>AY452770</td>
</tr>
<tr>
<td>Genus <em>Neodorippe</em> Serène and Romimohtarto, 1969</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Neodorippe callida</em> (Fabricius, 1798)</td>
<td>1</td>
<td>Yinggehai, Hainan</td>
<td>00085</td>
<td>AY452775</td>
</tr>
<tr>
<td>Genus <em>Paradorippe</em> Serène et Romimohtarto, 1969</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Paradorippe granulata</em> (De Haan, 1841)</td>
<td>2</td>
<td>East China Sea</td>
<td>D64B–1</td>
<td>AY452773</td>
</tr>
<tr>
<td></td>
<td></td>
<td>East China Sea</td>
<td>D64B–1</td>
<td>AY452774</td>
</tr>
<tr>
<td><em>Paradorippe polita</em> (Alcock and Andersocn, 1894)</td>
<td>2</td>
<td>East China Sea</td>
<td>C37B–9</td>
<td>AY452776</td>
</tr>
<tr>
<td></td>
<td></td>
<td>East China Sea</td>
<td>C37B–9</td>
<td>AY452777</td>
</tr>
</tbody>
</table>

* The specimens were identified by Professor Chen H L, IOCAS.

Additional sequences included the in- and outgroup analyses are obtained from Gen-Bank, including 7 species of genus *Sesarma* (family Grapsidae), and *Daphnia pulex* is used as outgroup. Sequences used in the analyses and their GenBank numbers are as follows.

16S rDNA sequences: Daphniidae *Daphnia pulex* NC–000844; Grapsidae *Sesarma aequatoriale* AJ225874; *S. sp.* AJ225871; *S. curacaoense* AJ225870; *S. crassipes* AJ225869; *S. reticulatum* AJ225867; *S. sulcatum* AJ225853; *S. rhizophorae* AJ225851.

#### 2.2 Total genomic DNA extraction, PCR, and sequencing

Total genomic DNA is isolated from muscle tissue of walking legs using a phenol-chloroform extraction (Kocher et al., 1989). Amplification of a fragment of the 16S rRNA gene is carried out by polymerase chain reaction (PCR) with the general primers 16Sar (5'-CGCCTGTATTATCCAAAAACAT-3') and 16Sbr (5'-CCGGTCTGAAGTCAGCAGT-3') in a PTC–100 Programmable Thermal Controller Cycler (MJ research, INC) in a 50-mm³ reac-
tion volume containing 5 mm$^3$ of 10 × PCR buffer, 4 mm$^3$ of MgCl$_2$ (2.5 mmol/dm$^3$), 4 mm$^3$ of dNTP (2.5 mmol/dm$^3$), 2 mm$^3$ of each primers (10 pmol/mm$^3$), 30.6 mm$^3$ of PCR-Grade water, 0.4 mm$^3$ of Taq polymerase (5 U/mm$^3$), and 2 mm$^3$ of genomic DNA. These primers, or modifications thereof, have been widely used to amplify a homologous portion of the 16S rRNA gene in many invertebrates (Xiong and Kocher, 1991; Simon et al., 1994; Cunningham et al., 1992; Huber et al., 1993; Avise et al., 1994; Black and Piesman, 1994), and a wide variety of vertebrates (Orti et al., 1996). The PCR temperature profile is 94 °C for 10 min followed by 34 cycles of 94 °C for 40 s, 50 °C for 40 s, 72 °C for 50 s. After the final cycle, samples are incubated for a further 10 min at 72 °C and then held at 4 °C prior to analysis. All the purified PCR products are sequenced in both directions with the same primers used in the amplification, which are completed by CASarray Co., Ltd. in Shanghai.

2.3 Phylogenetic analysis

Sequences are aligned with the sequence alignment program CLUSTALX1.81 (Thompson et al., 1997). The settings used are: pairwise parameters equal slow accurate, gap opening 12.00, gap extension 3.00; multiple parameters equal gap opening 12.00, gap extension 3.00, delaying divergent sequences 40%, DNA transition weight 0.50.

Sequence divergence is analyzed by using Jukes–Cantor distance, neighbor-joining (NJ) analysis and maximum parsimony (MP) analysis with the program MEGA (Kumar et al., 2001). Statistical significance of groups within inferred trees is evaluated by the bootstrap method with 1 000 replicates.

2.4 Time estimation

The process of time estimation is mainly based upon four steps (Nei and Kumar, 2002). Firstly, the 16S rDNA tree is constructed by using the neighbor-joining and maximum parsimony methods. Secondly, rate constancy (Takezaki et al., 1995) is tested by using the two-cluster test method (Takezaki, 1998). Thirdly, linearized tree is built under the premise of rate constancy. At last, the substitution rate is determined for Sesarma species (Schubart et al., 1998) utilizing the geological event of closure of the Panama Land Bridge and then used to estimate the divergence time for the Dorippe lineage.

3 Results

3.1 16S rDNA tree

The aligned 16S rDNA dataset contains 17 sequences (13 species) and 552 positions on the average. Of all the positions, 259 are variable and 174 are parsimony informative sites. Neighbor-joining and maximum parsimony methods render the same tree topologies (see Fig. 1).

3.2 Two-cluster test result

The result of the two-cluster test at each interior node in Fig. 1 is listed in Table 2. At every node, the difference of the branch length is given in delta and its standard error is se. Q=17.319 027 is given, which is for a test of rate constancy that combines the rate difference for all the interior nodes under the root. So, the constancy of evolutionary rate is approved.

3.3 Divergence time and the linearized tree

The divergence time at Node B and Node E (Schubart et al., 1998) is 3.1 Ma ago, in accord with the well constrained dating of the geological closure of the Panama Land Bridge. The average rate of nucleotide substitutions per site is 0.255 4% per million years, and 0.279 0% per million years, respectively. Then, the mean rate 0.267 2% per million years is used to estimate
Fig. 1. Phylogenetic relationships of 5 species of Dorippinae and outgroup species based on 16S rDNA sequences. Tree topology is based on neighbor-joining (NJ) analysis with Jukes–Cantor genetic distances. Statistical significance of groups within inferred trees is evaluated by the bootstrap method with 1000 replicates. Numbers are bootstrap values from an NJ analysis (above lines) and an MP analysis (below lines). Bootstrap values of being less than 50% are not shown. *Daphnia pulex* is used to root the tree. The *Sesarma* species contain the trans-isthmian species used in calibration of the molecular clock. Node O represents the origin of the *Dorippe* lineage. Scale bar: genetic distance.

<table>
<thead>
<tr>
<th>Node</th>
<th>delta</th>
<th>se</th>
<th>Z</th>
<th>CP (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>0.003 114</td>
<td>0.003 115</td>
<td>0.999 544</td>
<td>67.78</td>
</tr>
<tr>
<td>B</td>
<td>0.003 513</td>
<td>0.005 861</td>
<td>0.599 458</td>
<td>44.48</td>
</tr>
<tr>
<td>C</td>
<td>0.006 202</td>
<td>0.004 631</td>
<td>1.339 355</td>
<td>81.64</td>
</tr>
<tr>
<td>D</td>
<td>0.000 651</td>
<td>0.004 176</td>
<td>0.155 863</td>
<td>11.92</td>
</tr>
<tr>
<td>E</td>
<td>0.000 29</td>
<td>0.005 522</td>
<td>0.052 599</td>
<td>3.98</td>
</tr>
<tr>
<td>F</td>
<td>0.001 626</td>
<td>0.007 338</td>
<td>0.221 579</td>
<td>17.42</td>
</tr>
<tr>
<td>G</td>
<td>0.000 484</td>
<td>0.000 484</td>
<td>0.999 23</td>
<td>67.78</td>
</tr>
<tr>
<td>H</td>
<td>0.005 777</td>
<td>0.004 297</td>
<td>1.344 625</td>
<td>81.98</td>
</tr>
<tr>
<td>I</td>
<td>0.000 000</td>
<td>0.000 000</td>
<td>NAN</td>
<td>NAN</td>
</tr>
<tr>
<td>J</td>
<td>0.010 649</td>
<td>0.015 826</td>
<td>0.672 859</td>
<td>49.72</td>
</tr>
<tr>
<td>K</td>
<td>0.005 334</td>
<td>0.004 059</td>
<td>1.313 986</td>
<td>80.98</td>
</tr>
<tr>
<td>L</td>
<td>0.002 241</td>
<td>0.013 147</td>
<td>0.170 454</td>
<td>13.50</td>
</tr>
<tr>
<td>M</td>
<td>0.020 151</td>
<td>0.014 915</td>
<td>1.350 996</td>
<td>82.30</td>
</tr>
<tr>
<td>N</td>
<td>0.018 945</td>
<td>0.023 091</td>
<td>0.820 461</td>
<td>58.78</td>
</tr>
<tr>
<td>O</td>
<td>0.063 559</td>
<td>0.030 103</td>
<td>2.111 382</td>
<td>96.52</td>
</tr>
</tbody>
</table>

Notes: $Z = \frac{\delta - \bar{\delta}}{\sigma / \sqrt{n}}$, CP equals 1 minus $P$ value.
the divergence time for all clades, and a linearized tree is constructed (Fig. 2).

![Diagram]

Fig. 2. Linearized tree of Dorippinae based on a portion of the mitochondrial ribosomal large subunit (16S rRNA) genes. The branch lengths are reestimated under the assumption of rate constancy.

4 Discussion

4.1 Relationships among subgroups of Dorippinae

All species of Dorippinae were assigned to the genus *Dorippe* Weber, 1795 before 1969. Serène and Romimohtarto (1969) revised the Indo–Malayan species of *Dorippe*, dividing that genus into 3 genera and 2 subgenera: *Dorippe* Weber, 1795, comprising the subgenus *Dorippoides*; *Neodorippe*, including the subgenus *Nobilum*; and *Paradorippe*. Subsequently, Holthuis and Manning (1981) described genera *Medorippe* and *Phyllodorippe* and raised all of the subgenera recognized by Serène and Romimohtarto (1969) to genus rank; Chen (1985) described genus *Philippidorippe*. After then, Holthuis and Manning (1990) totally recognized 17 species of Dorippinae, distributed among 8 genera, including their new genus *Heikea*, from the Indo–West Pacific region.

Our analysis strongly supports the recognition of the Dorippinae as a monophyletic subfamily. It is also clear that *Heikea* Holthuis and Manning, 1990 is closely related to *Neodorippe* Serène and Romimohtarto, 1969. The divergence time between them is 15.8 Ma. Genus *Dorippe* appears basal in the subfamily Dorippinae, diverging from other species 36.6 Ma ago.

4.2 Molecular clock

Although the rate of nucleotide substitution would never be the same for all evolutionary lineages, the extent of rate heterogeneity is usually moderate when relatively closely related sequences are used, so that we can obtain rough estimates of divergence time between species from molecular data. And because of a poor or nonexistent fossil record, geological events rather than first appearances of sister-taxa are most commonly used when determining the substitution rate.
For 16S rDNA sequence data, Sturmbauer et al. (1996) estimated an average rate of nucleotide substitutions per site million years of 0.45% for fiddler crab populations across the Isthmus of Panama (closure 3.1–3.5 Ma ago). Schubart et al. (1998) estimated a slightly lower rate of 0.33%–0.44% per million years. More recently, Wares (2001) estimated the rate of evolution at 0.34% per million years for chthalamid barnacles. In our study, this rate is 0.27% per million years, closer to that of mammals (0.2% per million years; Mindell and Honeycutt, 1990). Since few data are available in marine primitive crabs, we could not explain this deference.

Acknowledgements

This study was supported by the National Natural Science Foundation of China under contract Nos 30370186 and 40276044, and the Project of the Chinese Academy of Sciences under contract No. KSCX2-SW–101B. We are grateful to Mr. Wu Longtao, and all other laboratory mates, for their helpful advice during our experiment. We also thank Mr. Wang Shaoqing (MBMCAS), for his kind help in preparation of the crab material.

References


Fabricius J C. 1798. Supplementum Entomologiae Systematicae. Hafniae 572


Takezaki N. 1998. Lintrdos. Tokyo National Institute of Genetics, Japan


