第10卷第5期
2003年10月

中国水产科学
Journal of Fishery Sciences of China

Vol. 10 No. 5
Oct., 2003

中华绒螯蟹蜕皮抑制激素1(*MIH1*)基因的cDNA片段克隆和Northern印迹分析

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摘要:根据其他蟹类蜕皮抑制激素的氨基酸序列设计了简并引物,运用RT-PCR和RACE技术,从中华绒螯蟹(*Eriocheir japonica sinensis*)成体的蜕皮间期眼柄中,克隆了1条长1 145 bp的cDNA。该cDNA编码60个氨基酸,包括942 bp的3′端非翻译区。同源性分析结果表明,该cDNA编码的氨基酸序列和其他蟹类蜕皮抑制激素有较高的一致性,最高的一致性为64%,将获得的序列命名为中华绒螯蟹蜕皮抑制激素1基因(GenBank检索号:AY309062)。用Northern印迹分析的方法对处于各个发育阶段的胚胎及溞状幼体期该基因的表达进行了研究,结果表明,处于胚胎发育早期的卵裂期几乎没有检测到杂交信号,而胚胎发育的其他时期和溞状幼体期都检测到蜕皮抑制激素1基因的mRNA。中华绒螯蟹蜕皮抑制激素1基因部分cDNA序列的获得为进一步获得该基因的全长cDNA、研究其功能及阐明中华螯蟹蜕皮的分子机制奠定了基础。

关键词:蜕皮抑制激素基因;cDNA克隆;中华绒螯蟹;Northern印迹分析
中图分类号:Q959.223;Q785　　**文献标识码**:A　　**文章编号**:1005-8737-(2003)05-0353-06

甲壳类动物的生长过程伴随着周期性的蜕皮现象,这种现象是由蜕皮抑制激素(molt-inhibiting hormone, MIH)和蜕皮激素相互拮抗来调控的。经生化鉴定,MIH属于甲壳动物高血糖激素(crustacean hyperglycemic hormone, CHH)家族神经肽。CHH家族成员除了CHH、MIH外,还包括性腺抑制激素(gonad-inhibiting hormone, GIH)和大颚器官抑制激素(mandibular organ-inhibiting hormone, MOIH)[1]。MIH在眼柄X-器官的表达量最高,其次在脑,而在其他组织中几乎没有表达[2-4]。对胚胎发育各期*MIH*基因的表达研究表明,刀额新对虾(*Metapenaeus ensis*)的胚胎发育期没有*MIH*基因的表达[4],但斑纹蟳(*Charybdis feriatus*)孵化前的胚胎中*MIH*基因有较弱的表达[5]。这说明MIH不仅参与调节甲壳类成体的蜕皮过程,而且可能还有其他功能。

由于蜕皮是蟹类生活史中重要的过程,因此对蜕皮抑制激素基因的结构及功能的研究有助于对蜕皮现象的深入了解,进而为养殖过程中出现的中华绒螯蟹性早熟现象分子机理的阐明积累资料。目前已在多种蟹类获得了蜕皮抑制激素的氨基酸序列[6-8]。国内关于中华绒螯蟹蜕皮现象的研究多偏重于生理学、形态学等方面[9-11],分子水平的研究仅见1篇报道[12]。本研究运用RT-PCR和RACE技术,克隆了1条编码序列与其他蟹类蜕皮抑制激素一致性较高的中华绒螯蟹蜕皮抑制激素1基因(molt-inhibiting hormone 1 gene for *Eriocheir japonica sinensis* 或 *Ers-MIH1* gene)的部分cDNA序列,并用Northern印迹分析的方法对该基因在胚胎发育各个时期的表达进行了研究。中华绒螯蟹蜕皮抑制激素1基因部分cDNA序列的获得,为进一步获得该基因的全长cDNA、研究其功能及表达调控机制,进而阐明中华绒螯蟹的蜕皮及养殖过程中出现的性早熟现象的分子机理奠定了基础。

收稿日期:2002-11-11;　**修订日期**:2003-05-13.
基金项目:国家自然科学基金重点项目(30130040);教育部"211"工程资助项目.
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1 材料与方法

1.1 材料

1.1.1 动物材料 成体中华绒螯蟹采自江苏连云港川海水产养殖场;处于发育各时期的胚胎和溞幼Ⅲ期的中华绒螯蟹采自安徽河蟹原种场。

1.1.2 试剂 JM109 菌株和 PinPoint™ Xa-1 T-质粒载体、RNA marker、*Taq* DNA 聚合酶和 Wizard® *Plus* SV Minipreps DNA Purification System 购自 Promega 公司。RNeasy® Mini Kit 购自 QIAGEN 公司。THERMOSCRIPT™ RT-PCR System (THERMOSCRIPT™ RT 和 PLATINUM *Taq* DNA polymerase)为 LIFE TECHNOLOGIES 产品。DIG High Primer DNA Labeling and Detection Starter Kit Ⅱ、CSPD、Blocking Reagent、Anti-DIG-AP 购自 Boehringer Mannheim 公司。N-Hybond nylon membrane 购自 Amersham 公司。

1.2 方法

1.2.1 简并引物的设计 依据美洲黄道蟹(*Cancer magister*)、可口美青蟹(*Callinectes sapidus*)、三叶真蟹(*Carcinus maenas*)和斑纹蟳(*Charybdis feriatus*)4种蟹类 MIH 肽的氨基酸序列设计简并引物。选择成熟肽1-10、43-54位的2个保守区域并根据已知蟹类 *MIH* 基因密码子使用频率分别设计上游的简并引物:F1: 5′-AGAGTWATHAAYGAYGARTGTCC-3′和下游的简并引物 R48: 5′-CACACACCACASGAAGTCYTC-3′,预计扩增的片段大小为 163 bp。

1.2.2 总 RNA 的制备 取10只处于蜕皮间期的成体中华绒螯蟹,切下其眼柄,在解剖镜下切开外壳,取出 X-器官窦腺复合体,迅速浸入液氮中。中华绒螯蟹的胚胎发育经历4个特征分明的阶段,即卵裂期、原肠期、眼点期和心跳期[13]。分别取各个发育阶段的胚胎约 100 mg,溞幼Ⅲ期的幼体 100 mg(以全体取材),迅速浸入液氮中。将液氮中的材料取出,用 RNeasy® Mini Kit 提取总 RNA。通过1.2%甲醛琼脂糖凝胶电泳及紫外荧光光度计测定 OD_{260} 和 OD_{280} 值,分别检测总 RNA 的完整性和纯度。

1.2.3 RT-PCR 将 1 μg X-器官窦腺复合体总 RNA 加入到 10 μL 反应液(75 mmol/L KCl, 8 mmol/L $MgCl_2$, 25 mmol/L Tris-HCl, 10 mmol/L DTT, 5 μmol/L 随机引物, 1 mmol/L dNTPs, 20 U RNASEOUT™, 2.5 U THERMOSCRIPT™ RT)中,并于42 ℃反应 1 h,逆转录合成第一链 cDNA(1st cDNA)。反应结束后,85 ℃处理5 min,然后再加2 U *E. coli* RNase H,37 ℃温育20 min以降解 RNA。

将上述合成的第一链 cDNA 稀释10倍后取1 μL用作简并引物 F1 和 R48 PCR 扩增的模板。PCR 反应总体积为 30 μL,含 50 mmol/L KCl、3 mmol/L $MgCl_2$、10 mmol/L Tris-HCl、0.5 U *Taq* DNA 聚合酶、200 μmol/L dNTPs、引物各 50 pmol。反应程序为:95 ℃预变性 2 min,95 ℃ 10 s,52 ℃ 30 s,30个循环,72 ℃延伸 20 min。

1.2.4 PCR 产物的克隆和测序 PCR 产物纯化后与 PinPoint™ Xa-1 质粒载体相连,然后克隆测序。序列测定在 ABI 310 遗传分析仪上进行。

1.2.5 3′RACE 取 1 μg X-器官窦腺复合体总 RNA 作模板,用3′端通用引物 CDⅢ(5′-GGCCTCGCGACGACTACd$(T)_{17}$-3′)逆转录合成第一链 cDNA。反应体系中除了将随机引物换为引物 CDⅢ(3 μmol/L)外,其他同1.2.3;50 ℃反应 1 h。

根据简并引物 F1 和 R48 扩增获得的序列设计基因特异性引物 Ers FP(5′-AAACCTGATCGGGAATCGTGAC-3′)。取上述第一链 cDNA 为模板,用基因特异性引物 Ers FP 和3′端接头引物 Ap02(5′-G GCCTCGCGACGACTACTT-3′),进行 3′RACE PCR 扩增。反应程序如下:95 ℃预变性 2 min;95 ℃ 10 s,58 ℃ 20 s,72 ℃ 4 min,30个循环;72 ℃延伸 20 min。PCR 产物纯化后,克隆测序。

1.2.6 序列的同源性分析 运行 Blastp 程序(http://www.ncbi.nlm.nih.gov/BLAST),将获得的核苷酸序列对应的氨基酸在 Swissport 中搜寻同源序列。

1.2.7 Northern 印迹分析 首先将各个发育阶段的胚胎及溞幼Ⅲ期的幼体的总 RNA 用 DNase(RNase free)处理,以消除可能存在的基因组 DNA 污染,然后进行 RNA 转膜。将1.2.3中获得的 PCR 产物纯化后,按照试剂盒说明用随机六核苷酸引物制备地高辛标记的 cDNA 探针,然后按照试剂盒说明进行杂交。

2 结果

2.1 提取的总 RNA

从处于蜕皮间期的中华绒螯蟹眼柄 X-器官窦腺复合体提取的总 RNA 的 OD_{260}/OD_{280} 值为1.9,满足纯度要求。总 RNA 经1.2%的甲醛琼脂糖凝胶电泳后可见分别在1.8 kb、1.9 kb 和2.6 kb 处有3

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条rRNA带(图1),此结果与斑节对虾(*Penaeus monodon*)的总RNA电泳结果类似[14]。

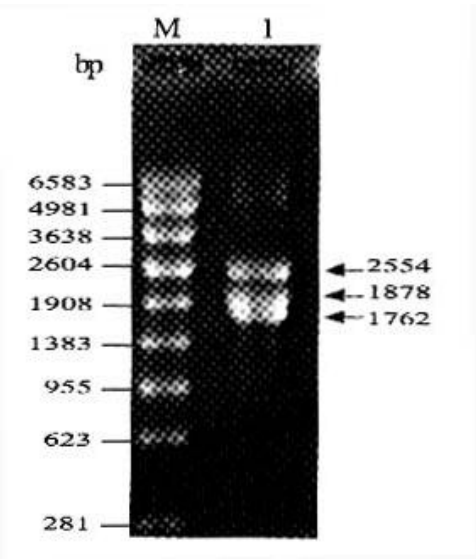


图1　中华绒螯蟹眼柄X-器官窦腺复合体总RNA甲醛琼脂糖凝胶电泳图　(M:RNA标记)

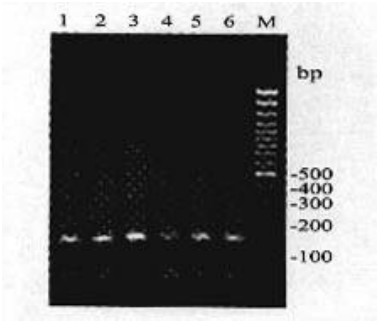
Fig.1　Formaldehyde agarose gel electrophoresis of total RNA isolated from X-organ sinus gland complex in the eyestalks of Chinese mitten crab　(M:RNA marker)

2.2　RT-PCR扩增产物的序列

简并引物F1和R48扩增产物的琼脂糖凝胶电泳结果表明获得了1条接近靶序列大小(163 bp)的条带(图2)。该条带纯化后克隆测序获得的序列进行Blastx分析的结果表明,该序列对应的氨基酸序列与其他蟹类MIH的一致性和相似性较高。其中,与食用黄道蟹(*Cancer pagurus*)、美洲黄道蟹和斑纹蝓的一致性分别为78%、76%和72%,相似性皆为87%。

2.3　3′RACE PCR扩增产物

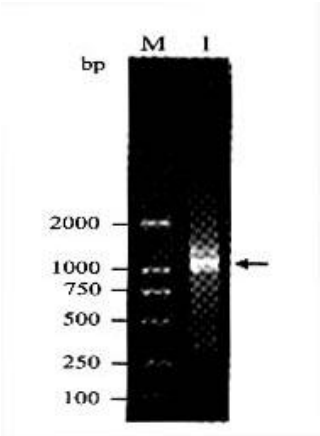
3′RACE PCR扩增产物的琼脂糖凝胶电泳结果显示获得了1条大于1 kb的主带(图3),该主带经克隆测序后获得1 145 bp的cDNA序列。该序列由203 bp的编码区(编码67个氨基酸)和942 bp的3′端非翻译区组成,加尾信号AATAAA位于距离终止密码子(TAA)下游897 bp处(图4)。Blastx分析结果表明,编码区对应的氨基酸序列与食用黄道蟹、美洲黄通蟹和三叶真蟹MIH的一致性和相似性最高,其一致性分别为64%、62%和61%,相似性皆为77%。这说明该序列可能是中华绒螯蟹*MIH*基因的3′端cDNA序列,命名为*Ers-MIH1*基因。*Ers-MIH1*基因的GenBank检索号为AY309062。



M:100 bp DNA Ladder Plus; 1－6: Amplification products of degenerate primers

图2　简并引物扩增产物的电泳图

Fig.2　Electrophoresis of amplification products of degenerate primers



M:DNA Marker DL 2 000; 1: Products of 3′RACE

图3　3′RACE PCR产物

Fig. 3　Products of 3′RACE PCR

2.4　Northern印迹分析

甲醛琼脂糖凝胶电泳结果(图5)表明,提取的胚胎各期和溞幼Ⅲ期的总RNA都有完整的rRNA带(图5－A),说明总RNA完整性较好。地高辛标记的*Ers－MIH1*基因的cDNA探针与发育各阶段胚胎和溞幼Ⅲ期的总RNA杂交图谱见图5－B。

图5－B表明,处于胚胎发育早期的卵裂期几乎没有检测到杂交信号,而胚胎发育的其他时期和溞幼Ⅲ期都明显检测到*MIH1*基因的表达。此外,胚胎发育的后3个时期,在较大分子量处也有杂交信号,而溞幼Ⅲ期没有出现大分子量的杂交信号。

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AAACCTGATCGGGAATCGTGACATCTACAAGAAGGTGGACTGGATCTGTGAGGACTGCGCCAACATCTTCCGCATCGACGGTCTGGGCATGCTCTGCAGG   100
  N  L  I  G  N  R  D  I  Y  K  K  V  D  W  I  C  E  D  C  A  N  I  F  R  I  D  G  L  G  M  L  C  R
AAGAACTGCTTTAGGAACATCGACTTCCTGTGGTGTGTGTACGCCTCGGAACGGCACGCACAGAAGGACGACCTCACGCGTTACGTCAGCATCCTCGGGC   200
  K  N  C  F  R  N  I  D  F  L  W  C  V  Y  A  S  E  R  H  A  Q  K  D  D  L  T  R  Y  V  S  I  L  G
AATAACTCCTGGGTGCCCCACCTGCCGCTCCCTCCCTCCCTCCCCGGGGTATCTGCCGCTGCTGGAGATGCCCCGAGGGTGAAGCCGATGTCCGGGAAGC   300
Q  *
GCCTTGAAGTGAAGAGTGAAGTGCGTCTCTCCAGCAGCTGTCCCTCCCTCTCTGGCTGCTCTTCGTCCGTGGTGTAAGAATCCAGCGGCTAAAGCCTTGG   400
TTCAGTCTCTCGCGAGGAGCTTTGATTGGCCTCCGTTCACCTCCATTCAGTCGCTTGTCTCTGTTTAGCTCCGTTCACGCCTTCGTCTATTGTTCAGCCG   500
TTCACCTTCCGTTCACCTCGTTTACCCACAAAGCTCCACTTGTCAGTGCTTTCCTGGGTCCTCCAGAAAGGTCTTGCATATCAGGGACACTATTTTAGCA   600
TTGGGAATTGGAGGTAACAGAGTATAGGAAAAGACAATTACATTTTTGTTTGTGTACATGGACGAGTATTCGAATGTAGAAGGCTGCTGTAGTAGTTGTA   700
ATAGTAGCTGTAGTATATAGGAGGAGGAGGAGGAGCAGAAGGAGGAGCAGGAGTGGAAAAGGAAGAAGAGAGGAGGAAGAAGGTATTTGAAAGTAGAAAT   800
AAGTTGAAAGACATCTCACTGAAACAGTATTCTCAAAACTCTAGTCAACTCTAAAGCTGTTTGAGGGTTTTCTCTTTATGAATGTGTATGTGTGATCGTA   900
CTCTTGTTTATTTGCATTCTTGGTTAGTGTTGAATCATTCCTTTGTGTGTGAGTGTGTGTATGTCAACTTCCTTTGTTATTTTGTTTCACTTTGTGTTAT  1000
CCTCTCTCGTTTTGTCCTAATTTATTGAGGTTGTAGTATTTATTGAAATCTGAGCCGCTGTGTTCTTCCACGTCTTAGAATTAATTGTGGCTGATAAAAA  1100
AAATAAAGAGAAACGAAAGAGC (A) n                                                                          1145
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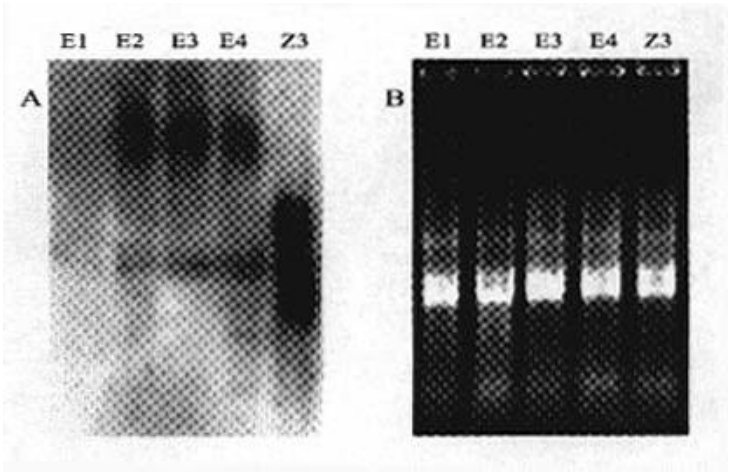
图4 *Ers－MIH1* 基因的部分 cDNA 的核苷酸序列及推导的氨基酸序列

Fig. 4 Nucleotide and deduced amino acid sequences of the partial cDNA of *Ers－MIH1* gene

AATAAA: polyadenlation signal



E1：卵裂期，E2：原肠期，E3：眼点期，E4：心跳期，Z3：溞幼Ⅲ

E1: egg cleavage stage; E2: gastrula stage; E3: eye pigment stage; E4: heart-beating stage; Z3: zoaea Ⅲ

图5 A 河蟹胚胎和溞幼Ⅲ期总 RNA 甲醛琼脂糖凝胶电泳；B *Ers－MIH1* 基因在河蟹胚胎和溞幼Ⅲ期的 Northern 印迹分析

Fig. 5 A Formaldehyde agarose gel electrophoresis of total RNA isolated from each developmental stage of embryos and zoaea Ⅲ of Chinese mitten crab; B Northern blot analysis of *Ers－MIH1* gene with the embryos at every stages and zoaea Ⅲ of Chinese mitten crab

3 讨论

3.1 RT-PCR 和 RACE

自 1988 年 Frohman 等[15]建立 cDNA 末端快速扩增(RACE)法以来，RACE 技术不断得到改进，现已广泛地应用于 cDNA 序列研究中。在甲壳类高血糖激素家族神经肽的研究中，已有文献采用此方法[2,5,8]。本研究运用 RACE 方法获得的中华绒螯蟹 *MIH1* 基因的 cDNA 序列覆盖成熟肽部分区域和 3′端非翻译区。中华绒螯蟹 *MIH1* 基因部分 cDNA 序列的测定，为该基因全长 cDNA 序列的获得及进一步获得该基因的基因组序列和该基因的功能研究奠定了基础。

在根据氨基酸序列设计的简并引物扩增中，引物的设计尤为重要，可通过分析密码子的使用频率，有效降低引物的简并度。我们在研究中先后合成了 7 对引物，仅本文报道的这对引物成功获得了中华绒螯蟹 *MIH1* 基因的部分 cDNA 序列，该对引物的简并度是最小的。

3.2 关于 Northern 印迹分析

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对胚胎发育各阶段 *MIH* 基因表达情况的研究表明,在刀额新对虾(*Metapenaeus ensis*)的胚胎期没有检测到 *MIH* 基因的表达[4],但在斑纹蝲孵化前的胚胎中检测到 *MIH* 基因弱的表达[5]。本研究的Northern印迹分析结果表明,在中华绒螯蟹胚胎发育的原肠期 *MIH1* 基因已开始表达。由于CHH家族神经肽在结构上具有相似性,该家族的有些成员呈现出功能的多样性[16-18],由此推测 *Ers-MIH1* 基因编码的蛋白可能在中华绒螯蟹胚胎发育过程中发挥某种作用,具体功能则有待于进一步研究。杂交中出现的大分子量杂交信号,推测可能是未加工的前体mRNA。

3.3　基因的结构分析

根据结构的不同CHH家族神经肽可分为CHH和VIH(vitellogenin inhibiting hormone)2组,其中VIH组又可分为RIH(reproduction inhibiting hormone)和MIH 2个亚组[19]。从结构组成来看,CHH组神经肽由信号肽、前体相关肽(CHH precursor-related peptide,CPRP)和成熟肽3部分组成,该组共有的3个二硫键的位置为$C^{7}-C^{43}$、$C^{23}-C^{39}$和$C^{26}-C^{52}$。而VIH组(包括MIH)神经肽仅由信号肽和成熟肽2部分组成,缺少CPRP,且该组共有的3个二硫键的位置为$C^{7}-C^{44}$、$C^{24}-C^{40}$和$C^{27}-C^{53}$[20]。由于本文报道的序列和王在照等[12]报道的序列皆为部分序列,因此无法从编码蛋白的结构组成上判断所克隆的基因是否是CHH家族的 *MIH* 基因。但从氨基酸组成来看,本研究获得的序列与其他蟹类MIH的一致性和相似性更高,最高的一致性和相似性分别为64%和77%。而王在照等[12]报道的序列与虾类的一致性和相似性较高,最高的分别达到71.8%和84.5%,但与蟹类的一致性和相似性分别只有42%和67%左右[12]。我们认为必需将结构特征和功能分析结合起来才能最终确定CHH家族各成员。

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Molecular cloning and Northern blot analysis of a cDNA fragment of molt-inhibiting hormone gene 1 from *Eriocheir japonica sinensis*

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Abstract: Degenerate primers were designed according to the MIH amino acid sequences of *Cancer magister*, *Callinectes sapidus*, *Carcinus maenas* and *Charybdis feriatus*. Using reverse transcription PCR (RT-PCR) and rapid amplification of cDNA end(RACE) techniques, a cDNA sequence of 1 145 bp was obtained from the eyestalks of *Eriocheir japonica sinensis*. The cDNA encodes a polypeptide of 60 amino acids and includes 3′ terminal untranslated region of 942 bp. The homology analysis revealed that the amino acid sequences coded by the cDNA obtained showed higher identities with the MIH of the four species above, and the highest identity was 64%. So it is named *Ers-MIH1* gene (molt-inhibiting hormone 1 gene for *Eriocheir japonica sinensis*, i.e. *Ers-MIH1* gene) temporarily. The accession number of *Ers-MIH1* gene in GenBank is AY309062. Northern blot analysis was performed with the embryos at each developmental stage and the Chinese mitten crab zoaea Ⅲ to study the expression of this gene. The results showed that hybridization signal was hardly detected in the early developmental embryos at egg cleavage stage, while the mRNA of the *Ers-MIH1* gene was detected in the embryos at other developmental stages and in the Chinese mitten crab zoaea Ⅲ. By obtaining the partial cDNA sequence of *Ers-MIH1* gene, cloning of the full-length cDNA sequence of this gene, a further study on its function and the elucidation of the molecular mechanism in molting of Chinese mitten crab will be possible.

Key words: molt-inhibiting hormone gene; cDNA cloning; *Eriocheir japonica sinensis*; Northern blot analysis

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第10卷第5期
2003年10月

中国水产科学
Journal of Fishery Sciences of China

Vol. 10 No. 5
Oct., 2003

Sequence analysis on mitochondrial *16S rRNA* gene in *Fenneropenaeus chinensis*

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Abstract: *Fenneropenaeus chinensis* is of economical importance in marine fishery of China. This paper deals with the PCR amplification and sequence analysis of mitochondrial *16S rRNA* gene fragment of its wild and cultured populations (CP1, CP4, CP5 and CP6). There was no difference among the *F. chinensis* individuals in the sequence of 520 bp obtained in this research. The A, T, G and C contents of the sequence were 171 bp (32.88%), 176 bp (33.85%), 104 bp (20.00%) and 69 bp (13.27%) respectively, and the content of AT was higher than that of GC. Furthermore, 413 bp of the fragment was used to discuss the phylogenetic relationship of 12 Penaeidae shrimps belonging to genera *Penaeus*, *Farfantepenaeus*, *Fenneropenaeus*, *Marsupenaeus and Litopenaeus* using *Alpheus armillatus* as the outgroup. The molecular phylogenetic tree constructed by neighbor-joining method showed that there were two large shrimp clusters. *F. chinensis* and *P. monodon* were clustered first, and then clustered with *M. japonicus*; another large shrimp cluster was *Farfantepenaeus* cluster with *Litopenaeus* cluster consisting of their individuals. The results also indicated that there was a closer genetic relationship between genus *Farfantepenaeus* and genus *Litopenaeus*; also between *F. subtilis* and *F. paulensis*, *L. schmitti* and *L. setiferus*.

Key words: *Fenneropenaeus chinensis*; *16S rRNA*; sequence analysis; Penaeidae; phylogeny
CLC number: Q959.223 **Document code**: A **Article ID**: 1005-8737-(2003)05-0359-06

Fenneropenaeus chinensis, which belongs to the family Penaeidae, is mainly distributed in Bohai Sea and Yellow Sea in China. This species is of economical importance in both fishing and aquaculture in China[1]. To protect the natural population from over-exploitation, artificially produced seeds have been released to enhance the natural stocks since 1985. Considering the importance of this species, genetic information is vital to the design and implementation of sound fisheries management strategies and sustainable development of aquaculture.

There have been many studies on population structure and phylogeny in shrimps[2-12]. In this study, a part of mitochondrial *16S rRNA* gene was amplified by PCR, and the fragment sequenced was used for analysis of the genetic variations between wild and cultured populations (CP1, CP4, CP5 and CP6). Furthermore, phylogenetic relationships of 12 Penaeidae shrimps were discussed using the related gene sequences downloaded from the GenBank.

Received date: 2003-03-25; **Revised date**: 2003-05-19.
Foundation item: Supported by projects under the Major State Basic Research Development Program (G1999012009).
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1 Materials and Methods

1.1 Materials

The samples of wild *F. chinensis* population were collected from coastal waters of Rizhao, Shandong Province, and the first, forth, fifth and sixth generation of cultured populations (CP1, CP4, CP5 and CP6) were from Jiaonan, Rizhao, Jimo and Rizhao, respectively. The shrimp specimens were initially fro-

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zen in liquid nitrogen (−196 ℃) and subsequently maintained at below −20 ℃. Two individuals of each population were used for sequence analysis.

1.2 Genomic DNA extraction

For genomic DNA extraction, a small piece of muscle (about 100 mg) was ground in a glass homogenizer and incubated for 2 h at 37 ℃ in a 700 μL STE solution (100 mmol/L NaCl, 10 mmol/L EDTA, 50 mmol/L Tris-HCl, pH 8.0) containing 50 μL 20% SDS and 15 μL 10 mg/μL proteinase K. The DNA was isolated using a standard phenol/chloroform extraction protocol and collected by ethanol precipitation[13].

1.3 PCR amplification

The target fragment of *16S rRNA* gene was amplified by using primers L2510 5′-CGCCTGTTTAACAAAAACAT-3′ and H3059 5′-CCGGTCTGAACTCAGATCATGT-3′[12]. Double-stranded PCR amplifications were conducted in a biometra thermocycler using an initial 90 s denaturation at 94 ℃ followed by 35 cycles of 30 s at 94 ℃ for denaturation, 45 s at 49 ℃ for annealing, 1 min at 72 ℃ for extention, and a final 10 min extension at 72 ℃. The amplification reactions were carried out in a 25 μL volume in each Ready-To-Go (Pharmacia Biotech.) tube that contained approximate 100 ng template DNA 1 μL, 1 μL of each primer and 22 μL sterile deionized H_2O. In all PCR amplifications, negative controls consisting of template-free reactions were used to detect DNA contamination. A total of 2 − 3 μL of each PCR product was used for 1.5% agarose gel electrophoresis for verifying the amplified fragment length with a DNA marker DL2 000 [Takara Biotechnology (Dalian) Co, Ltd], and then was visualized with ethidium bromide under ultraviolet light.

1.4 Sequencing

The amplified products were electrophoresed on a 1.5% agarose gel and purified with Wartson's gel extraction kit. These purified products were used as the template DNA for cycle sequencing reactions performed using Dye Terminator Cycle Sequencing FS Ready Reaction Kits (Applied Biosystems), and run on an ABI 377 DNA sequencer (Perkin-Elmer Corp). Double strands were sequenced and the primers used for sequencing were the same as those for PCR amplification.

1.5 Data analysis

The sequences were edited and aligned by the DNASTAR software (DNASTAR, Inc). Phylogenetic and molecular evolutionary analyses were conducted using MEGA version 2.1[14]. A 413 bp homologous fragment of 12 Penaeidae shrimps was adopted for phylogenetic analysis.

2 Results and Discussion

A 560 bp segment of *16S rRNA* gene was amplified and sequenced from the 10 individuals of five different populations in *F. chinensis*. The sequences of 520 bp except the primers were obtained and there was no sequence variation among those individuals (Fig. 1). The A, T, G and C contents in the sequence were 171 bp (32.88%), 176 bp (33.85%), 104 bp (20.00%) and 69 bp (13.27%), respectively.

```
GTCTATATGATTGTTATATAAAGTCTAGCCTGCCCACTGATTTAGTTTAA 50
AGGGCCGCGGTATATTGACCGTGCGAAGGTAGCATAATCATTAGTCTTTT 100
AATTGAAGGCTTGTATGAATGGTTGGACAAAAAGTGAGCTGTCTCAATTA 150
TAATAATTGAATTTAACTTTTAAGTGAAAAGGCTTAAATGAATTAAGGGG 200
ACGATAAGACCCTATAAAGCTTGACAATAATTTAATTATACTATCAATTG 250
TTAGTGTAACTTGGTTTTAATTAATATTTGTTACGTTGGGGCGACGGGAA 300
TATAACAAGTAACTGTTCTTAAATATTTAATAACAAATATAATTGGAAAA 350
CTAGCATGATCCTCTATTAGTGATTAAAAGATTAAGTTACTTTAGGGATA 400
ACAGCGTAATCTTCTTTGAGAGTCCTCATCGACAAGAAGGTTTGCGACCT 450
CGATGTTGAATTAAGGTATCCTTATGATGCAGCAGTTGTAAAGGAAGGTC 500
TGTTCGACCTTTAAATCCTT 520
```

Fig. 1 ***16S rRNA*** **gene sequences (520 bp) of** ***F. chinensis***

图1 中国对虾*16S rRNA*基因序列(520 bp)

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The gene fragment of 413 bp was analyzed to examine the phylogenetic relationship of 12 Penaeidae shrimp species (*Litopenaeus stylirostris*, *L. vannamei*, *L. schmitti*, *L. setiferus*, *Farfantepenaeus subtilis*, *F. paulensis*, *F. duorarum*, *F. brasiliensis*, *F. aztecus*, *Fenneropenaeus chinensis*, *Marsupenaeus japanicus*, *Penaeus monodon*). *Alpheus armillatus* was selected as the outgroup.

Figure 2 shows the variation sites of *16S rRNA* sequences among the 13 species above. It was clear that AT content (66.5% ~ 72.0%) was higher than GC content, and there was a little difference of A, T, G and C among five genus in contents (Table 1).

Table 1　Base composition of *16S rRNA* gene segment of 12 Penaeidea shrimp species

表 1　12 种对虾 *16S rRNA* 基因片段的碱基组成

Species 种类	Abbreviation 简写	Content/% 含量					Total length/bp 片段总长	Accession number 注册号
		A	T	G	C	A+T		
Litopenaeus stylirostris	L. st	33.3	34.2	20.1	12.4	67.5	412	AF255056
L. vannamei	L. va	33.7	32.8	20.6	12.9	66.5	412	AF192089
L. schmitti	L. sc	34.5	33.3	20.4	11.9	67.8	412	AF192086
L. setiferus	L. se	34.6	33.2	20.3	11.9	67.8	413	AF192078
Average of *Litopenaeus*		34.0	33.4	20.4	12.3	67.4		
Farfantepenaeus subtilis	Fa. s	33.3	32.3	22.1	12.4	65.6	412	AF192076
F. paulensis	Fa. p	34.0	31.1	21.6	13.3	65.1	412	AF192060
F. duorarum	Fa. d	34.0	33.0	21.1	11.9	67.0	412	AF192056
F. brasiliensis	Fa. b	34.7	33.0	20.4	11.9	67.7	412	AF192054
F. aztecus	Fa. a	35.0	32.3	20.4	12.4	67.3	412	AF192052
Average of *Farfantepenaeus*		34.2	32.3	21.1	12.4	66.5		
Fenneropenaeus chinensis	Fe. c	34.6	33.7	19.6	12.1	68.3	413	
Marsupenaeus japanicus	M. ja	33.4	33.9	20.5	12.2	67.3	410	PJA388112
Penaeus mondon	P. mo	36.6	35.4	20.6	7.5	72.0	413	AF125383
Alpheus armillatus	A. ar	29.0	35.0	24.6	11.4	64.0	403	AF501644

Table 2　Genetic distances calculated among 12 Penaeidea shrimps based on Kimura's two parameter method

表 2　用 Kimura 双参数法计算 12 种对虾的遗传距离

Species 种类	L. st	L. va	L. sc	L. se	Fa. s	Fa. p	Fa. d	Fa. b	Fa. a	Fe. c	M. ja	P. mo	A. ar
Litopenaeus stylirostris	—												
L. vannamei	0.0427	—			Average distance among *Litopenaeus* species = 0.055 3								
L. schmitti	0.0691	0.0746	—										
L. setiferus	0.0664	0.0691	0.0098	—									
Farfantepenaeus subtilis	0.0915	0.0884	0.1261	0.1227	—								
F. paulensis	0.1055	0.1110	0.1376	0.1342	0.0615	—	Average distance among *Farfantepenaeus* species = 0.064 2						
F. duorarum	0.0914	0.1026	0.1199	0.1166	0.0747	0.0639	—						
F. brasiliensis	0.0888	0.1058	0.1143	0.1110	0.0863	0.0641	0.0616	—					
F. aztecus	0.0912	0.0967	0.1167	0.1135	0.0671	0.0509	0.0586	0.0534	—				
Fenneropenaeus chinensis	0.1052	0.1081	0.1048	0.1074	0.1379	0.1312	0.1289	0.1052	0.1135	—			
Marsupenaeus japanicus	0.1112	0.1083	0.1404	0.1341	0.1286	0.1139	0.1141	0.0970	0.1111	0.0937	—		
Penaeus mondon	0.1917	0.2012	0.2042	0.2003	0.2175	0.2238	0.1977	0.1879	0.2071	0.1574	0.1823	—	
Alpheus armillatus	0.2615	0.2919	0.2945	0.3015	0.3032	0.2869	0.2762	0.2872	0.2872	0.2850	0.2868	0.3599	—

Genetic distance among the 12 Penaeidae shrimp species was calculated based on Kimura's two parameter method. The results showed that the distance between genus was bigger (Table 2). From the molecular phylogenetic tree constructed by neighbor-joining method, two large clusters were obtained: *F. chinensis* and *P. monodon* were clustered first, and then clustered with *M. japonicus*; another large shrimp cluster

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was *Farfantepenaeus* cluster and *Litopenaeus* cluster by their individuals. The results also indicated that the genetic relationship was close between genus *Farfantepenaeus* and *Litopenaeus*; also between *F. subtilis* and *F. paulensis*, *L. schmitti* and *L. setiferus* (Fig. 3).

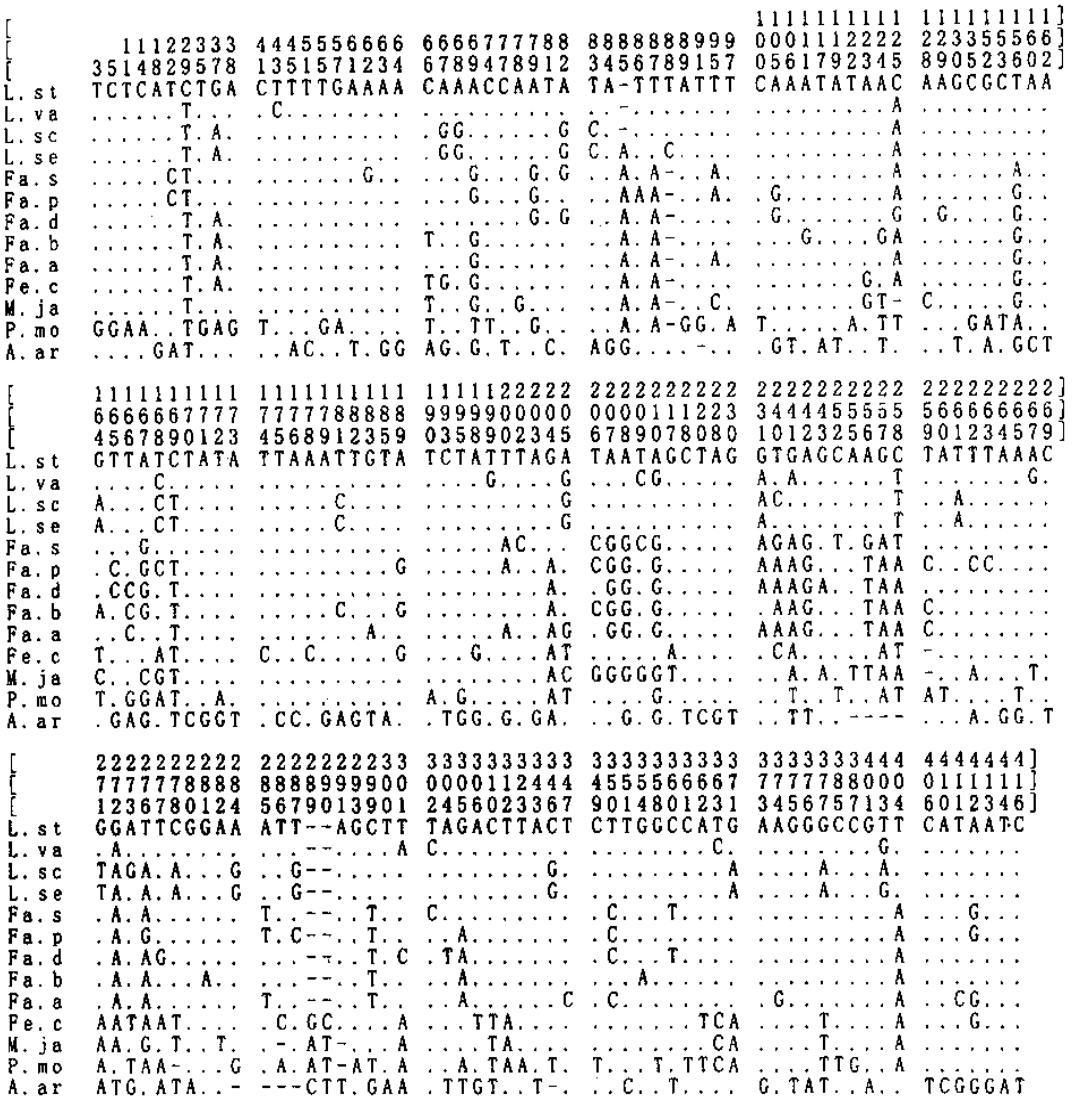

Fig. 2 Variation sites in 413 bp segment of *16S rRNA* gene among 12 Penaeidea shrimp species

图2 12种对虾*16S rRNA*基因片段(413 bp)的变异位点

Mitochondrial DNA has been extensively used in fishery study with features such as its compactness, maternal inheritance and fast evolutionary rate compared to nuclear DNA[15-17]. COI gene has been detected among 15 shrimp species[2,8]. Qiu et al. obtained the results that there were high polymorphism among 17 individuals of 4 populations in *F. chinensis* by using the same segment of *16S rRNA* gene[9]. Quan et al. amplified and sequenced 535 bp of *16S rRNA* gene and found no variation among the sequences of 24 *F. chinensis* individuals[10]. Our results were quite different from Qiu et al. 's. The difference of the results between ours and Quan et al. 's was because we cut the

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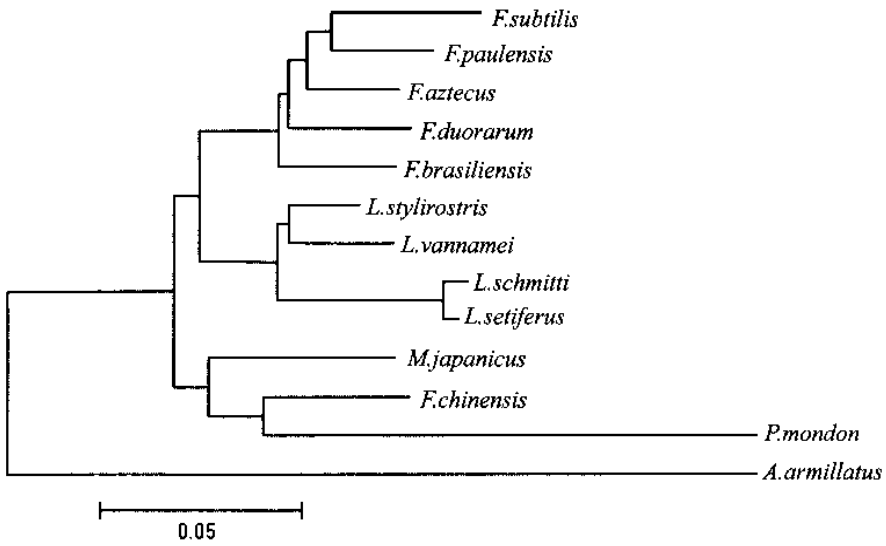

Fig. 3　Neighbor-joining molecular phylogenetic tree of 12 Penaeidea shrimps using *Alpheus armillatus* as the outgroup
图 3　以环斑鼓虾为外群构建的 12 种对虾分子系统树

two primers of the *16S rRNA* gene segment completely[9-10]. *16S rRNA* gene sequence (413 bp) was used to compare with other 11 Penaeidae shrimps. The results above indicated that the *16S rRNA* gene fragment is very conservative in *F. chinensis*. These results suggested that *16S rRNA* gene could not be used to study population genetics of *F. chinensis*, but it should be useful for phylogenetic analysis of Penaeidae shrimps. The further work on other genetic markers of *F. chinensis* populations such as microsatellite may be more desirable.

Acknowlegements

The authors wish to express many thanks to Dr. He Pingguo in University of New Hampshire (USA) for his assistance in correcting the manuscript.

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中国对虾线粒体 *16S rRNA* 基因序列分析

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摘要: 中国对虾(*Fenneropenaeus chinensis*)是我国海洋渔业重要的经济虾种之一。本研究以相应引物对野生群体和人工培育第1代、第4代、第5代、第6代群体(CP1、CP4、CP5、CP6)的各2个个体的线粒体 *16S rRNA* 基因片段进行了 PCR 扩增和序列测定分析。分析结果表明, 测得的 *16S rRNA* 基因片段长为 520 bp, 10 个中国对虾个体间无碱基差异, 其 A、T、G、C 含量分别为 171 bp (32.88%)、176 bp (33.85%)、104 bp (20.00%)、69 bp (13.27%), AT 含量明显高于 GC 含量。利用其中长度为 413 bp 的同源序列, 以环斑鼓虾(*Alpheus armillatus*)为外群探讨了对虾科(Penaeidae)中的对虾属、美对虾属、明对虾属、囊对虾属和滨对虾属 5 个属(*Penaeus*, *Farfantepenaeus*, *Fenneropenaeus*, *Marsupenaeus*, *Litopenaeus*) 12 种对虾间的系统关系。以 NJ 法构建的分子系统树显示可将以上 12 种虾类分为两个大群: 中国对虾和斑节对虾先聚到一起后与日本对虾聚成一大群; 美对虾属、滨对虾属个体各聚成 1 支后聚成一大群, 最后与中国对虾等聚到一起。同时可见, 小褐美对虾(*F. subtilis*)与保罗美对虾(*F. paulensis*)、南方滨对虾(*L. schmitti*)与白滨对虾(*L. setiferus*)种间的亲缘关系较近。

关键词: 中国对虾; *16S rRNA*; 序列分析; 对虾科; 亲缘关系

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