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硕士学位论文

日本囊对虾热休克蛋白 HSP60 基因克隆表达、多态性与耐热性状的关系

Molecular cloning, expression of heat shock protein 60
(HSP60) gene in *Marsupenaeus japonicus* and the association
of its polymorphisms with thermal tolerance

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摘要

日本囊对虾(*Marsupenaeus japonicus*)是我国重要经济养殖虾类,但在南方地区存在着高温夏季死亡率较高的养殖难题。因此,急需了解日本囊对虾的耐热性状及耐温的分子机制,为此,我们开展了日本囊对虾 MjHSP60 基因的克隆、表达分析及其多态性与耐热性状相关性的研究,获得了如下主要结果:

1. MjHSP60 基因克隆

在日本囊对虾 MjHSP60 cDNA 序列基础上克隆到了该基因 DNA 全序列。日本囊对虾 MjHSP60 基因全长 4055 bp,结构与拟穴青蟹一致,均由 8 个外显子及 7 个内含子构成,且对应的外显子长度极其相近,序列相似度达 94%,而内含子长度存在较大差异。

2. MjHSP60 mRNA 在日本囊对虾幼体阶段的表达

利用 qRT-PCR 分析发现 MjHSP60 在幼体发育阶段均有表达,包括:卵、无节幼体、溞状幼体、糠虾幼体及仔虾,说明其在日本囊对虾幼体发育中有重要作用。但各发育阶段表达量存在显著差异,总体上,从卵到糠虾幼体阶段的 MjHSP60 平均表达水平高于仔虾期,但表达水平呈现较大波动,且各期最低表达水平均出现在从一种形态转变到另一种形态的过渡期。推测其早期发育阶段 MjHSP60 表达量的变化与日本囊对虾形态变化、生活习性的转变等有关。

3. 日本囊对虾耐热性状研究

耐热性状在日本囊对虾种内与其他虾类种间都存在差异。首先,日本囊对虾种内不同大小的个体间耐热性存在差异,其体重与耐热性之间呈显著负相关($P < 0.05$),特别是日本囊对虾仔虾($< 0.01\text{g}$)的耐热性(UTT)显著高于 1~5g 日本囊对虾($P < 0.05$)。另外,日本囊对虾的耐热性状与其它虾类物种之间也存在明显差异,日本囊对虾仔虾耐热性低于凡纳滨对虾但高于脊尾白虾。

4. 日本囊对虾 2 种形态变异类型遗传结构的分析

以台湾澎湖、福建诏安、广西北海、海南三亚 4 个群体 2 种形态变异类型日本囊对虾为实验对象,利用直接测序法共筛选获得 42 个单核苷酸多态性位点(SNP),其中 25 个 SNP 位点位于内含子区,17 个位于外显子区,13 个为同义突变,4 个为非同义突变。4 个地理群体平均观测杂合度(H_o)为 0.1508~0.2444,平均期望杂合度(H_e)为 0.1493~0.2633,表明群体遗传多样性水平较低。属于同种形

态变异类型各群体间的 F_{st} 值均小于 0.05, 遗传分化不显著; 而属于不同种形态变异类型各群体间 F_{st} 值则较大, 在 0.2564~0.3471 之间, 表明遗传分化程度很高, 结合 N_m 值(0.100374~0.208228)分析表明有限的基因交流是 2 种形态变异类型群体发生遗传分化的主要原因。日本囊对虾群体间的遗传距离 (D_A) 为 0.0088~0.1492, 基于遗传距离构建的 UPGMA 聚类图显示, 北海、三亚群体聚为一支, 澎湖、诏安群体聚为另一支, 2 个分化类群的地理分布与 Tsoi 的 2 个类型相符合。

5. 日本囊对虾 MjHSP60 基因 SNP 与耐热性的相关性

由于不同形态变异类型日本囊对虾的耐热性不同, 本研究以 Variety I 群体作为热敏感群体, Variety II 群体作为耐热群体, 进行 MjHSP60 基因 SNP 与耐热性的相关分析。检测发现有 3 个位点(-213、-1541、-3061)在 Variety I 中的分布偏离 Hardy-Weinberg 平衡(HWE), 2 个位点(-212、-2060)在 Variety II 中的分布偏离 HWE, 而位点-2011 在两个群体中的分布均偏离 HWE, 其余位点在两个群体中的分布均符合 HWE。除了 2 个位点不同基因型(-282、-2011)在两个群体中的差异不显著外, 其余位点在两群体中的差异均达到显著($P < 0.05$)或极显著($P < 0.01$)水平, 表明在这些位点中可能找到与日本囊对虾耐热性相关的潜在 SNP 标记。

另外, 各多态性位点之间存在不同程度的连锁不平衡, 提示该基因存在与日本囊对虾耐热性相关的单体型。对 31 种频率 > 3% 的单体型在 2 种形态变异类型中的分布频率进行分析发现, 15 种单体型在 Variety II 中的分布频率显著高于 Variety I, 并且其中 2 种单体型为 Variety II 所特有, 提示在这些单体型中可能找到与日本囊对虾耐热性相关的潜在遗传标记; 而 Variety I 也形成了 11 种优势单体型, 其中 5 种为 Variety I 所特有。

利用 PCR-RFLP 方法检测发现, MjHSP60 基因外显子区的同义突变位点 -3289A/G 两种基因型与日本囊对虾 UTT 显著相关($P < 0.05$), 进一步验证了 HSP60 基因 SNP 位点与日本囊对虾耐热性的相关性。

关键词: 日本囊对虾; HSP60; 耐热性; SNP

Abstract

Marsupenaeus japonicus is an important commercial species in China, however, high temperature has been a negative factor for *M. japonicus* farming in summer. Therefore, it's important to understand the thermal resistance and mechanism of heat resistance of *M. japonicus*. In the present study, a HSP60 gene from *M. japonicus* was cloned, the expression of MjHSP60 mRNA during early developmental stages was investigated, and the association of its polymorphisms with thermal tolerance was analyzed. The main results are shown as follows:

1. Cloning of MjHSP60 gene

The full length of MjHSP60 was successfully cloned with a length of 4055 bp based on the cDNA sequence, the gene structure was essentially no difference with that of *Scylla paramamosain*, including 8 exons and 7 introns, the length of corresponding exons were nearly the same and the exon sequences similarity was 94%, while the length of introns were obviously different.

2. The expression of MjHSP 60 during early development

As detected by qRT-PCR, MjHSP60 was expressed in all examined developmental stages in *M. japonicus* including eggs, nauplius, zoeas, mysis and post larvae. During its early developmental stages (from eggs to mysis), the mRNA level of MjHSP60 was higher than post larvae on average, but underwent major fluctuations and the troughs corresponded to the transition periods between each two stages, which implied MjHSP60 is related to a number of early developmental and physiological functions of *M. japonicus*.

3. Thermal tolerance of *M. japonicus*

Significant differences in thermal tolerance were observed both within *M. japonicus* and in different species. Firstly, the thermal tolerance differed greatly among different sizes of *M. japonicus*, the body weight showed a negative correlation with UTT ($P < 0.05$), and the UTT of juveniles (< 0.01 g) was significantly higher than that of the group weighing 1~5 g ($P < 0.05$). Secondly, the thermal tolerance of *M. japonicus* was lower than *Litopenaeus vannamei* and higher than *Exopalaemon carinicauda* ($P < 0.05$).

4. Genetic structure analysis of two morphologically similar varieties (I and II) of *M. japonicus*

Two varieties of 4 wild stocks of *M. japonicus*, i.e. Penghu (PH) stock in Taiwan, Zhaoan (ZA) stock in Fujian province, Beihai (BH) stock in Guangxi province, Sanya (SY) stock in Hainan province were used as experimental subjects, 42 SNP were detected by direct sequencing, including 25 SNP in the introns and 17 SNP in the exons, among which 13 SNP were synonymous mutations, and 4 SNP were nonsynonymous mutation. The mean observed heterozygosity (H_o) ranged from 0.1508 to 0.2444 and the mean expected heterozygosity (H_e) ranged from 0.1493 to 0.2633, indicating a low level of genetic diversity in the 4 wild stocks. The pair-wise genetic differentiation (F_{st}) within the same variety were lower than 0.05, suggesting the genetic differentiation was inconspicuousness; while F_{st} between two varieties ranged from 0.2564 to 0.3471, indicating a high level of genetic differentiation. The value of gene flow (N_m) between two varieties were small (0.100374~0.208228), declaring the limited gene exchange between two varieties was the main cause of genetic differentiation. The genetic distance (D_A) among stocks ranged from 0.0088 to 0.1492. Cluster analysis based on D_A indicated that BH and SY stocks were clustered as one cluster while PH and ZA stocks were gathered in another group. The geographic distributions of these two differential groups were consistent with the two varieties.

5. The correlation of MjHSP60 SNP and thermal tolerance of *M. japonicus*

On account of the different thermal tolerance between two varieties of *M. japonicus*, Variety I was employed as heat sensitive stock and Variety II was employed as heat resistant stock to study the correlation between MjHSP60 SNP and thermal tolerance. Statistical analysis revealed that the genotypic frequencies showed deviation from Hardy-Weinberg equilibrium (HWE) at loci -213, -1541 and -3061 in Variety I stock, -212 and -2060 in Variety II stock, and -2011 in both stocks, while the rest loci were in HWE. There were significant differences in genotypic frequencies between two varieties at all loci except for loci -282 and -2011, indicating a significant association with the thermal tolerance of *M. japonicus*.

Linkage disequilibrium was found between the multiple loci of MjHSP60 gene,

and 31 haplotypes with frequency above 3% were identified. Among them the frequencies of 15 haplotypes in Variety II stock were significantly higher than that in Variety I, indicating their significant associations with thermal tolerance of *M. japonicus*. In the contrast, 11 haplotypes in Variety I stock were significantly higher than that in Variety II stock.

Two different genotypes at loci -3289A/G were found to be significantly correlated with the UTT of *M. japonicus* by PCR-RFLP ($P < 0.05$), which confirmed the association between MjHSP60 SNP and thermal tolerance.

Key Words: *Marsupenaneus japonicus*; HSP60; thermal tolerance; SNP

缩略语中英文对照

| 缩写 | 英文 | 中文 |
|----------|--|------------|
| Amp | ampicillin | 氨卡青霉素 |
| ANOVA | analysis of variance | 方差分析 |
| bp | base pair | 碱基对 |
| cDNA | complementary DNA | 互补脱氧核糖核酸 |
| CTMax | critical thermal maxima | 临界高温 |
| D_A | Genetic distance | 遗传距离 |
| DDW | double distilled water | 双蒸水 |
| dNTP | deoxyribonucleoside triphosphate | 脱氧核糖核苷三磷酸 |
| EB | ethidium bromide | 溴化乙啶 |
| EDTA | ethylene diamine teraacetic acid | 乙二胺四乙酸 |
| F_{is} | inbreeding coefficient | 近交系数 |
| F_{st} | genetic differentiation index | 遗传分化指数 |
| H_e | expected heterozygosity | 期望杂合度 |
| H_o | observed heterozygosity | 观测杂合度 |
| HWE | Hardy-Weinberg equilibrium | 哈迪-温伯格平衡 |
| HSP | heat shock protein | 热休克蛋白 |
| kDa | kilodalton | 千道尔顿 |
| LB | Luria-Bertani medium | LB 培养基 |
| MAS | marker assisted selection | 标记辅助育种 |
| mRNA | messenger ribonucleic acid | 信使 RNA |
| NCBI | National Center for Biotechnology Information | 美国国家生物信息中心 |
| ORF | open reading frame | 开放阅读框 |
| PCR | polymerase chain reaction | 聚合酶链式反应 |
| RT | reverse transcription | 反转录 |
| SDS | sodium dodecyl sulfate | 十二烷基硫酸钠 |
| SNP | single nucleotide polymorphism | 单核苷酸多态性 |

缩略语中英文对照

| 缩写 | 英文 | 中文 |
|-------|-----------------------------------|------------------|
| TAE | Tris-acetic acid-EDTA buffer | Tris-乙酸 EDTA 缓冲液 |
| TBE | Tris-boric acid-EDTA buffer | Tris-硼酸 EDTA 缓冲液 |
| Tris | Tris (hydroxymethyl) aminomethane | 三(羟甲基)氨基甲烷 |
| UPGMA | unweighted pair-group method with | 非加权组平均法 |
| UTR | untranslated region | 非翻译区 |
| UTT | upper thermal tolerance | 耐热性 |

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