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# 黄颡鱼卵巢 P-450arom 基因的克隆及组织表达

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**摘要**:P-450 芳香化酶(P450arom)是催化雄激素生物合成雌激素的关键酶。本研究采用 RT-PCR 和 RACE(Rapid Amplification of cDNA Ends)法,分离和克隆了黄颡鱼(*Pelteobagrus fulvidraco*)卵巢 P450 芳香化酶基因 HP450arom A,并使用荧光实时定量 RT-PCR 对其组织表达进行了分析。结果表明,HP450arom A cDNA 全长 1 914 bp[不包括 poly(A)],5′端非翻译区有 13 bp,3′端 362 bp[不包含 poly(A)],阅读框(Open Reading Frame, ORF)1 539 bp,翻译成 513 个氨基酸,计算的蛋白质分子量为 58.7 kD。同源性分析显示,HP450arom A 的氨基酸序列与其他鱼卵巢 P450arom 具有 60%～90%的同源性,与其他鱼脑 P450arom 为 57%～60%同源,与鸡卵巢和人胚盘 P450arom 则为 51%和 52%同源;但芳香化酶高保守区包括Ⅰ-螺旋区,芳香化酶特异保守区Ⅱ和血红素结合区Ⅲ和其他鱼芳香化酶相比同源性分别高达 68%～97%、78%～91%和 71%～100%。系统发育分析表明,HP450arom A 与鱼类卵巢 P450arom 属于同一分支,黄颡鱼和鲇鱼亲缘关系最近,这和传统的分类方法结论一致。荧光实时定量 RT-PCR 研究结果显示,HP450arom A 在前脑、下丘脑、脑垂体、精巢、肝脏中不表达,只在卵巢中表达。这说明 HP450arom A 的表达具有组织特异性,并可推测其对卵巢发育起着重要作用。与本室分离的 HP450arom B 在卵巢的表达量相比,卵巢中 HP450arom A 的表达量是 HP450arom B 的 18.7 倍。

**关键词**:黄颡鱼;P450 芳香化酶;cDNA 末端快速扩增法;系统发育关系;基因表达
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黄颡鱼(*Pelteobagrus fulvidraco*),在分类学上隶属于硬骨鱼纲、鲇形目、鲿科、黄颡鱼属。黄颡鱼是以肉食性为主的杂食性鱼类。其肉质细嫩,味道鲜美,小刺,多脂。黄颡鱼不但有滋补作用,而且还有一定的药用价值,深受消费者青睐,历来被视作高档鱼类。同时,黄颡鱼在日本、韩国、东南亚等国家也有很大的市场潜力,是出口创汇的优良品种。黄颡鱼在自然水域中生长速度慢,上市规格小,在一定程度上影响了市场发展。在生产上提高黄颡鱼产量与效益最有效的办法是利用黄颡鱼雌雄之间在生长方面存在的明显差异,进行单性养殖,黄颡鱼雄性比雌性生长快 30%左右[1]。但有关黄颡鱼性别决定的分子机理研究还未见报道。现有研究表明,P450 芳香化酶与性别形成和发育有关[2],在性别形成期使用芳香化酶抑制剂可以使基因型为雌性的鲑鱼、罗非鱼转变为功能性雄鱼[3-4]。至今,已有鲇鱼(*Ictalurus punctatus*)[5]、金鱼[6]、罗非鱼(*Oreochromis niloticus*)[7-8]、斑马鱼(*Danio rerio*)[9]、赤点石斑鱼(*Epinephelus akaara*)[10]等的芳香化酶基因被分离和克隆,并研究了该基因与性别的关系。本研究室在分离了黄颡鱼脑 HP450arom B(GenBank accession number: AY649789)之后,在黄颡鱼卵巢中又分离到了 P450 芳香化酶基因 HP450arom A,并对其序列进行比较和分析,同时应用荧光实时定量 RT-PCR 研究了该基因在雌雄、鱼脑、肝脏及性腺的表达,旨为今后进一步研究该基因在黄颡鱼性别形成、卵巢发育中的表达提供基础依据。

## 1 材料与方法

### 1.1 材料

**1.1.1 实验动物** 基因分离用雌性黄颡鱼 2 尾,体长 9.5 cm 左右,体重 90 g 左右,所取组织为Ⅲ期卵巢;实时定量 RT-PCR 用黄颡鱼雌雄各 3 尾,体长 5.7～7.5 cm,体重 66～76 g,性腺发育为Ⅱ期,购

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**1.1.2 试剂** 抽提 RNA 用 Trizol Reagent (Promega)、反转录酶 M-MLV、Rnase H、TdT 酶、胶回收试剂盒，均购自 Takara；*Taq* 酶购自 Promega，pUCm-T 载体购自上海生工生物工程技术有限公司；SYBR 荧光定量 RT-PCR 测定试剂盒购买自 Takara。

**1.1.3 仪器** eppendorf Mastercycler personal PCR 仪，荧光定量 PCR 仪 Roche Lightcycler 1.2。

**1.1.4 引物** 用于 PCR 反应的所有引物如下，其碱基位置根据 GenBank 序列(GenBank accession number AY871802)确定。P1、P2、P3 根据已知鱼类 P450arom 的保守序列，使用 CodeHop 原理[11]设计；P4、P5 根据 P1、P2 分离的片段设计的用于卵巢芳香化酶基因 3′RACE 的特异引物；P6、P7、P8 是根据 P2、P3 引物分离的片段设计的用于卵巢芳香化酶基因 5′RACE 的引物；P9、P10 是用于定量 RT-PCR 的 HP450aromA 引物；引物 P13、P14 是用于定量 RT-PCR 的 β-actin 的引物。所有引物均由 Takara 合成，其中 R = A + G，N = A + C + T + G。

P1 碱基 927 - 953
5′-CAGTGTGTGTTGGAGATGGTGATCG-3′；
P2 碱基 1 382 - 1 354
5′-CTTCATCATCACCATGGCTATGTGCTT-3′；
P3 碱基 297 - 322
5′-CGGGTGTGGATCAACGGNGARGARAC-3′；
P4 碱基 997 - 1 099
5′-GCTATTGAAACAGAACGCAGAGG-3′；
P5 碱基 1 328 - 1 347
5′-GGTTCCGGTCCTCGTTCCTG-3′；
P6 碱基 860 - 850
5′-AGTTCAGGTGGTCAAGTTTT-3′；
P7 碱基 435 - 416
5′-ATTCCCTGTTCGTTCATCCC-3′；
P8 碱基 428 - 407
5′-GTTCGTTCATCCCGAGACACTG-3′；
P9 碱基 1 118 - 1 133
5′-AACGAATCTTTGCGCT-3′；
P10 碱基 1 366 - 1 349
5′-AGCTATGTGTTTACCAAC-3′；
P13 碱基 678 - 692
5′-ACTTCGAGCAGGAGAT-3′；
P14 碱基 906 - 890
5′-ACAGTGTTGGCATACAG-3′。

## 1.2 方法

**1.2.1 总 RNA 的抽提** 取新鲜卵巢，用 *Trizol* Reagent 抽提总 RNA。用变性琼脂糖凝胶电泳溴化乙啶染色显示 28 s 和 18 s，以检测 RNA 的完整性。

**1.2.2 RT-PCR 分离 HP450arom A**

(1) 保守片段的分离 取 5 μg 从卵巢抽提的总 RNA，以 dT-Ap [dT-AP，5′-CTGATCTAGAGGTACCGGATCC$(T)_{16}$-3′]为引物，用 *M-MLV* 根据使用说明进行 RT 反应，然后用 10% RT 液，使用引物 P1 和 P2 扩增 P450arom 450 bp 左右的保守序列，PCR 反应体系总体积 25 μL，其中含 5 μL 10× 反应缓冲液，2 μmol/L 氯化镁，200 μmol/L dNTP，引物各 0.4 μmol/L，2.5 U *Taq* 酶。反应条件 94 ℃ 3 min，然后 30 循环 94 ℃ 1 min，58 ℃ 1 min，72 ℃ 1 min，最后 72 ℃ 10 min，4 ℃ 保存；PCR 产物用 1.2% 的琼脂糖凝胶电泳分离，切胶，使用胶回收试剂盒回收，用 T－载体克隆，送测序。同理，使用 P3 和 P2 扩增得到 1 100 bp 左右的片段，退火温度使用 56 ℃，72 ℃ 延伸用 1 min 30 s。根据这些序列设计特异的 3′ RACE 和 5′ RACE 引物，分离扩增 P450arom 的 3′和 5′端序列。

(2) 3′RACE 方法 用 5 μg 总 RNA 以 dT-Ap [dT-AP，5′-CTGATCTAGAGGTACCGGATCC$(T)_{16}$-3′]为引物，根据使用说明用 M-MLV 进行 RT 反应，然后用 RT 液的 10%，以 AP[AP，5′-CTGATCTAGAGGTACCGGATCC-3′]和 3′RACE 特异引物 P4 进行 PCR 反应，PCR 总体积 25 μL，反应体系同上，反应条件 94 ℃ 3 min，然后 28 循环 94 ℃ 1 min，58 ℃ 1 min，72 ℃ 1 min，最后 72 ℃ 10 min，4 ℃ 保存；为增加扩增效率及扩增的特异性，把上述 PCR 液稀释 10 倍，取 2 μL 作模板，用引物 AP 和 3′RACE 第 2 个特异引物 P5 进行再扩增，退火温度为 55 ℃，扩增液用 1.2% 的琼脂糖凝胶电泳分离，回收，克隆，送测序。

(3) 5′RACE 方法 原理参照文献[12-13]，用 5 μg 总 RNA，以 5′RACE 第 1 个引物 P6，根据使用说明用 M-MLV 进行 RT 反应，然后加 RnaseH，分解 mRNA，用 DNA 回收试剂盒(Takara)回收 cDNA，去除多余的 dNTP，引物等；再用 TdT 酶在 cDNA3′端加 poly(A)，用试剂盒回收加了 poly(A)尾的 cDNA，以此为模板，用 5′RACE 第 2 个引物 P7 及 dT-AP(同 3′RACE)为引物，进行 PCR，反应体系组成同 3′RACE，PCR 液稀释 10 倍，取 2 μL 为模板，

用第 3 个引物 P8 及 AP，进行 PCR，反应体系组成同上，PCR 液用 1.2% 的琼脂糖凝胶电泳分离，回收，克隆，送测序。

（4）测序和序列分析　PCR 产物克隆到 pUCm-T 载体后，送上海开瑞生物芯片科技股份有限公司测序。序列分析用软件 DNATools 5.1，P450arom 的氨基酸同源性比较使用 ClustalW1.6[14]分析，比对结果用 PAUP（Phylogenetic Analysis Using Parsimony version 4.0，b2）[15]计算系统发育关系，用 Neighbor-Joining 法，重复 1 000 次，gap 处理为缺失。

（5）荧光定量 RT-PCR 测定 HP450arom A 的表达　取雌、雄黄颡鱼各 3 尾，分别抽提前脑、下丘脑、垂体、肝脏、卵巢或精巢总 RNA，以 β-actin 为内标，采用实时定量 RT-PCR 测定 HP450arom A 的表达。取 1 μg 总 RNA 使用 M-MLV（$H^-$）（试剂盒）进行 RT 反应，体系同前，RT 液稀释 10 倍，取 2 μL，分别用引物 9、10 和 11、12 使用 Ex *Taq* HS（试剂盒），$Mg^{2+}$ 5.0 μmol/L，引物浓度 0.2 μmol/L，20 μL 反应体系，以预变性：95 ℃、10 s 20 ℃/s，1 Cycle；PCR 反应：95 ℃、5 s、20 ℃/s、60 ℃ 20 s 20 ℃/s 50 Cycles；融解曲线分析：95 ℃、0 s、20 ℃/s，65 ℃、15 s、20 ℃/s，95 ℃、0 s、0.1 ℃/s。使用 Roche Light- Cycler 1.2 进行实时定量 PCR，根据融解曲线判别扩增是否特异，并在反应结束电泳检测，在卵巢的表达量根据实时定量 PCR 测得 Ct 值，计算 HP450arom A 和 β-actin 的表达量，以及 HP450arom A 与 β-actin 表达量的比值，并与 HP450arom B 在卵巢的相对表达量进行比较。

## 2　结果与分析

### 2.1　黄颡鱼卵巢 P450arom 的分离

取黄颡鱼卵巢组织的 RNA，采用 RT-PCR 和 RACE 法，分离了芳香化酶基因的保守序列以及 3′端和 5′端序列（图 1），把上述序列拼接得到了卵巢中芳香化酶基因 HP450arom A 全序列（GenBank accession number：AY871802），该 cDNA 总长 1 914 bp，其中阅读框 1 539 bp，翻译 513 个氨基酸，测算的蛋白质分子量为 58.7 kD，3′非翻译区 362 bp（不包括 poly（A）），5′非翻译区 13 bp（图 2）。

### 2.2　序列分析

**2.2.1　同源性比较**　使用 ClustalW 1.6 分析 HP-450arom A 氨基酸序列与鲇鱼[5]、金鱼卵[6]、斑马鱼卵[10]、黄鳝卵、鲇鱼脑[5]、金鱼脑[6]、斑马鱼脑[10]、黄颡鱼脑、鸡卵巢[16]、人胎盘[17]等 P450arom 氨基酸序列的同源性。结果表明，黄颡鱼 HP450arom A 的氨基酸序列与鲇鱼卵同源性最高达 90%，金鱼卵 71%、斑马鱼卵 73%、黄鳝卵 60%、鲇鱼脑 59%、黄颡鱼脑 58%、金鱼脑 59%、斑马鱼脑 58%、鸡卵巢

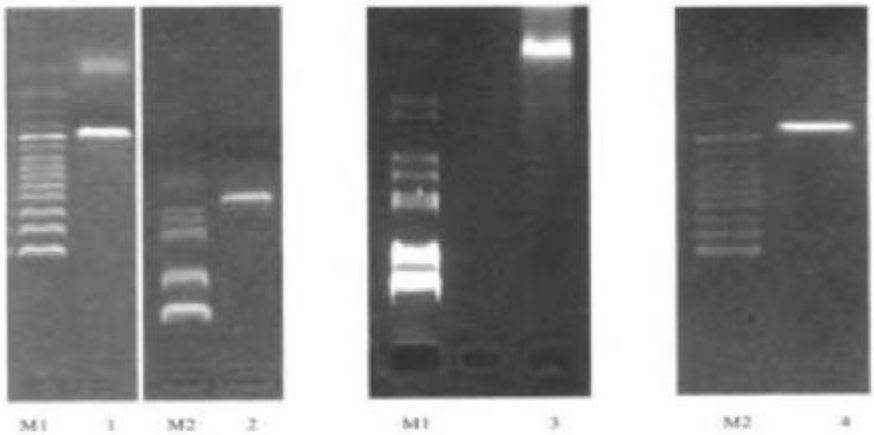



图 1　PCR 扩增图

M1：GeneRuler™ 100 bp DNA Ladder Plus；M2：λDNA/*EcoR* Ⅰ + *Hind* Ⅲ marker

1：引物 1、2 扩增条带；2：引物 3、2 扩增条带；3：3′RACE 引物 5、AP 扩增条带；4：5′RACE 引物 8、AP 扩增条带。

Fig.1　Results of PCR amplification

M1：GeneRuler™ 100 bp DNA Ladder Plus；M2：λDNA/*EcoR* Ⅰ + *Hind* Ⅲ marker

1：Product of amplification with primers 1 and 2；2：Product of amplification with primers 3 and 2；3：Product of amplification with 3′RACE primer 5 and AP；4：Product of amplification with 5′RACE primer 8 and AP.

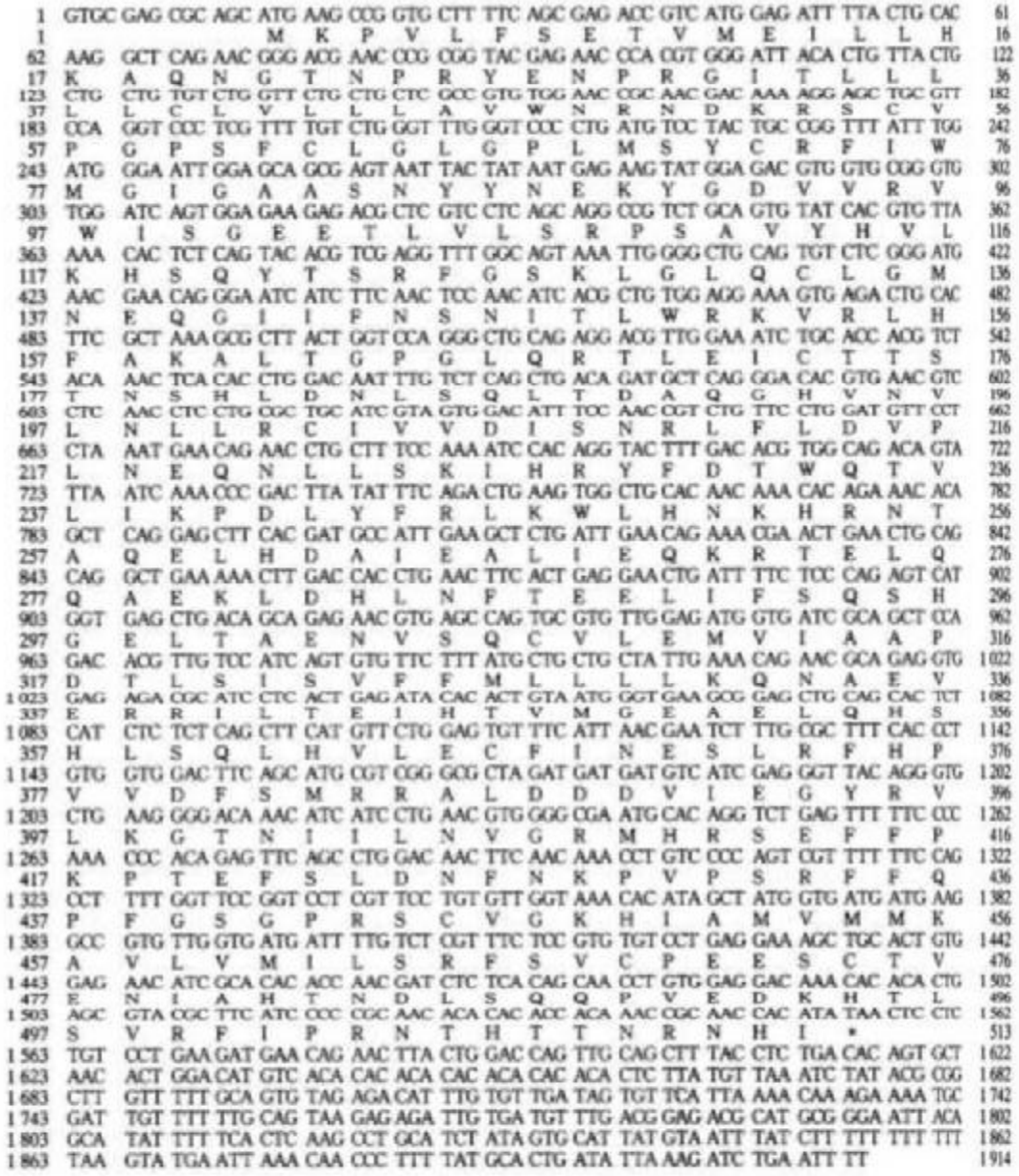


图2 黄颡鱼卵巢 HP450arom A 全长 cDNA 序列及翻译的氨基酸序列
(其中阅读框 1 539 bp 翻译 513 个氨基酸)

Fig.2 Nucleotideanddeducedamino acid sequence of yellow catfish (*Peltobagrus fulvidraco*) ovarian P450arom cDNA.
(The 1 539 bp ORF encodes a protein of 513 amino acids in length)

51%、人胎盘 51%。但芳香化酶高保守区包括Ⅰ-螺旋区,芳香化酶特异保守区Ⅱ和血红素结合区Ⅲ和其他鱼芳香化酶相比同源性分别高达 68%～97%,78%～91%和 71%～100%(图 3)。使用点突变方法研究人 P450arom 酶催化活性发现位点 I142、E310、P316、D317、T318、R443、C445(对应 HP450arom A)对酶活性起着重要作用[18],对比分析发现 HP450arom A 在这些位点均与其他芳香化酶一致(图 3),这进一步证明了这些位点对该酶催化活性的重要性。

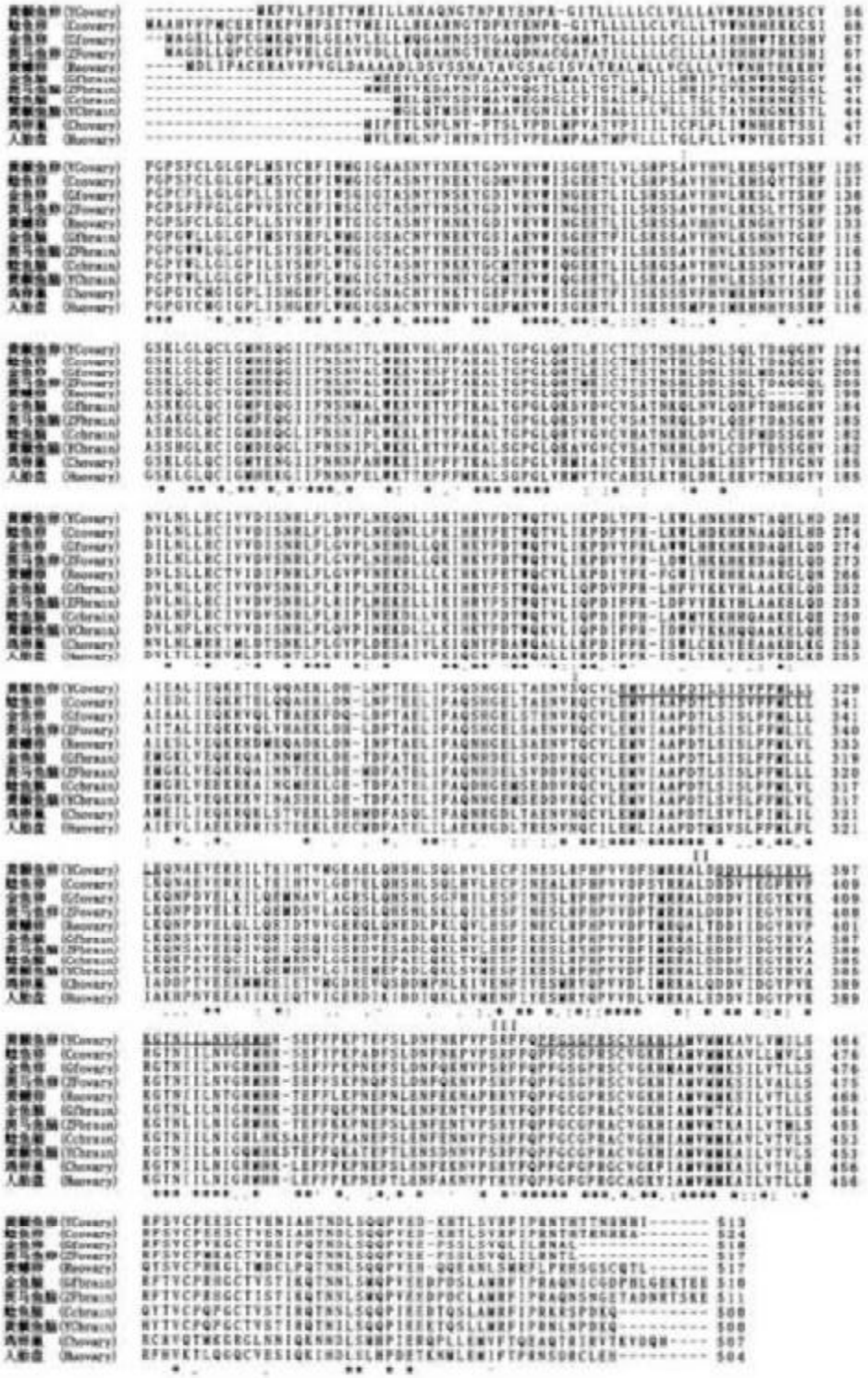


图 3　黄颡鱼卵巢 HP450arom A 氨基酸序列与鲇鱼等其他脊椎动物 P450arom 氨基酸序列的比较

Fig. 3　Alignment of yellow catfish ovarian HP450arom A amino acid sequence with sequences of other animals

图3注:图3中包括鲇鱼卵(Q92111),金鱼卵(AAC14013),斑马鱼卵(AAK00643),黄鳝卵(AY583785),金鱼脑(AAB39408),斑马鱼脑(AAK00642),鲇鱼脑(AAL14612),黄颡鱼脑(AY649789),鸡卵巢(A31916),人胎盘(Q4Hu19)。序列中高度保守的片段用下划线指示,并用罗马字表示,其中Ⅰ-螺旋区(Ⅰ),芳香化酶特异的保守区(Ⅱ),血红素结合区(Ⅲ)。氨基酸一致的用"*"表示,相似的用"."表示,对酶催化活性起重要作用的位点用黑体字表示。

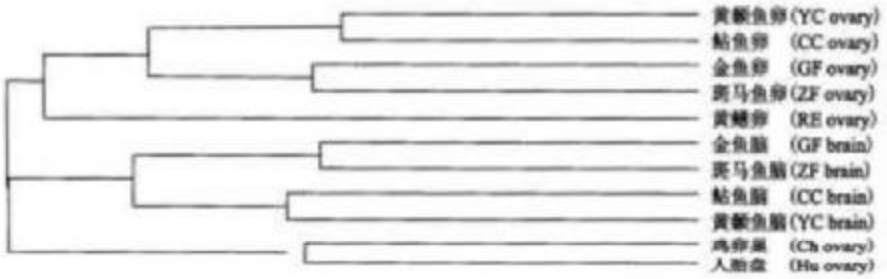
Explanation of Fig. 3: Channel catfish ovarian P450arom (Q92111), goldfish ovary (AAC14013), zebra fish ovary (AAK00643), rice field eel ovary (AY583785), goldfish brain-derived P450arom (AAB39408), zebrafish brain (AAK00642), channel catfish brain (AAL14612), yellow catfish brain (AY649789), chicken ovary (A31916), and human placenta (Q4Hu19). Regions of high homology are underlined and indicated by Roman numerals: I-helix (Ⅰ), an aromatase-specific conserved region (Ⅱ), and heme-binding region (Ⅲ). Identical and similar amino acids are marked by asterisks and dots, respectively. Amino acids known to be essential for catalytic functions are marked by bolds.

CC—Channel catfish; Ch—Chicken; GF—Golden fish; Hu—Human; RE—Rice eel; YC—Yellow catfish; ZF—Zebra fish

**2.2.2 系统发育分析** 使用上述比对结果,用PAUP软件计算系统发育关系,在总共539个位点中,185个位点由于处于变化较大的区域而被排除在系统发育关系之外,采用Neighbor-Joining法,重复1 000次,gap处理为缺失,构建了系统发育树(图4),从系统树可见HP450arom A属于鱼类卵巢P450arom一支,并与鲇鱼卵P450arom亲缘关系最近,这和传统分类结果一致,它们均属于鲇形目,另外,从系统树上可见黄颡鱼卵巢和脑P450arom分别属于鱼类卵巢和脑P450arom分支,和鲇鱼、金鱼、斑马鱼等一样,它们是由两个基因编码组成的。

### 2.3 HP450arom B在各组织的表达

实时定量RT-PCR结果显示,在雌雄黄颡鱼前脑、下丘脑、垂体、肝脏和精巢HP450arom A不表达,只有卵巢有表达(图5)。以β-actin为内标,测定HP450arom A在卵巢相对表达量并与HP450arom B的表达量相比较,结果卵巢中HP450arom A的表达量是HP450arom B的18.7倍(3尾雌鱼卵巢中这两基因表达量的比较的平均值)。




CC—Channel catfish; Ch—Chicken; GF—Golden fish; Hu—Human; RE—Rice eel; YC—Yellow catfish; ZF—Zebra fish

图4 根据黄颡鱼卵巢P450氨基酸序列用Njbootstrap构建的系统树

Fig. 4 Phylogenetic tree of yellow catfish ovarian P450arom proteins. The consensus tree was constructed by neighbor-joining bootstrap using PAUP4b10.

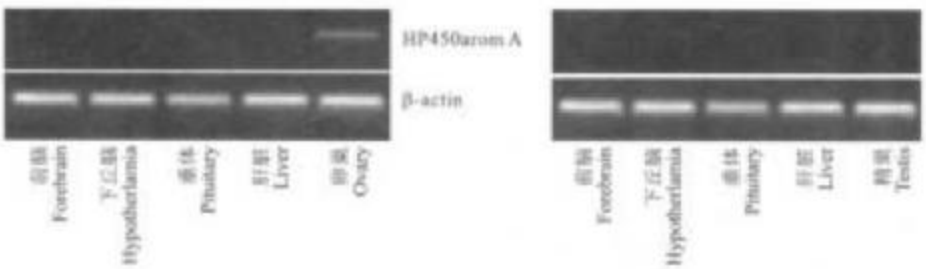



图5 HP450arom A在雌雄黄颡鱼各组织的表达

Fig. 5 Expression of HP450arom A in tissues of female and male yellow catfish

## 3　讨论

本研究从黄颡鱼卵巢中分离到了 HP450arom A，通过同源性及系统发育分析可知，它属于鱼类卵巢一支 P450arom，与本课题组之前所分离的 HP450arom B 显然是由不同基因编码的，其氨基酸相似性只有 58%，这印证了大部分鱼具有卵巢和神经两个芳香化酶基因[14]。从结构分析可见 HP450arom A 具有芳香化酶共有的保守区域Ⅰ－螺旋区、芳香化酶特异保守区Ⅱ和血红素结合区Ⅲ，并且这些区域的相似性较高，达 68%－100%。在对酶催化作用起重要作用位点的氨基酸分析中发现，I142、E310、P316、D317、T318、R443、C445 与其他芳香化酶均一致，比较保守，这些一方面说明了芳香化酶基因的这些位点在进化中的保守性，系统发育还揭示了 HP450arom A 与鲇鱼卵巢中的 P450arom 遗传距离最近，这和传统分类关系一致，它们同属于鲇形目，相比其他鱼亲缘关系最近。

本研究使用灵敏度高的荧光实时定量 RT-PCR 分析了 HP450arom A 在雌雄鱼各组织的表达，结果发现，除了在卵巢中检测到 HP450arom A 的表达外，在前脑、下丘脑、垂体、肝脏和精巢均没表达，这说明了该基因具有卵巢表达特异性，和在卵巢表达的 HP450arom B 的表达量比较发现，卵巢芳香化酶主要由 HP450arom A 表达，其表达量是 HP450arom B 的 18 倍左右。有关该基因在卵巢发育中的表达量以及表达的细胞定位研究正进行中。

对其他鱼类卵巢 P450arom 在各组织的表达也有研究，但结果不尽相同，使用 RT-PCR 在金鱼脑中检测卵巢 P450arom 的微弱表达[6]。使用 Northern 杂交研究斑马鱼卵巢 P450arom 的表达结果在脑、肝脏中没表达[9]，半定量 RT-PCR 研究赤点石斑鱼时发现卵巢 P450arom 在垂体、性腺有表达[10]。造成各研究结果不同可能与所研究鱼的种类、年龄、以及所处发育状态有关。但都一致的是 HP450arom A 和 HP450arom B 在脑和卵巢的表达量存在明显的组织特异性。

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# Molecular cloning of ovarian P450arom gene and its expression in male and female yellow catfish *Pelteobagrus fulvidraco*

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**Abstract**: The enzyme aromatase P-450(P450arom)can catalyse the conversion of androgen to oestrogen. A cDNA encoding P450arom was derived from yellow catfish (*Pelteobagrus fulvidraco*) ovary using RT-PCR and RACE. The cDNA was 1 914 bp with 13 bp 5′UTR, 362 bp 3′UTR[excluding poly(A)] and 1 539 bp ORF, which encoded 513 amino acids and had a predicted mol wt of 57.8 kD. The yellow catfish ovarian P450arom shared 60% - 90% sequence identity with ovarian aromatases of other four fish species, which were golden fish, channel catfish, rice eel and zebra fish, but shared only 57% - 60% with brain-derived aromatases of the four fishes, 51% and 52% with chicken ovarian and human placenta aromatases. But the percentage of identity/similarity was higher in the regions of high homology, including the I-helix, an aromatase-specific conserved region Ⅱ, and the heme-binding region Ⅲ, which were 67% - 96%, 78% - 86% and 78% - 100% respectively. Phylogenetic analysis of the P450arom gene family indicated the yellow catfish ovarian P450arom was clustered with the four fishes' ovarian P450arom, and yellow catfish was most close with channel catfish . This result is consistent with that of contradiction classification. The fluorescent real-time quantity RT-PCR analysis reveals that HP450arom A expresses only in ovary, but not in yellow catfish brain, liver nor testis. The comparison of HP450arom A and HP450arom B expressions in ovary indicates the expression level of HP450arom A is 18.7 times of that of HP450arom B.

**Key words**: *Pelteobagrus fulvidraco*; P450 aromatase; RACE; phylogenetic relatedness; gene expression

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# Isozyme analysis of *Sepia esculenta* (Cephalopoda: Sepiidae)

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**Abstract**: Horizontal starch gel electrophoresis was used to investigate the tissue-specific and genetic structure of *Sepia esculenta* Hoyle, 1885 (Cephalopoda: Sepiidae). The specimens were collected from the coast of Rizhao, Shandong Province of China. A preliminary screening for 19 isozymes in six kinds of tissues (eye, branchia, mantle muscle, buccal bulb muscle, branchial heart, liver) was carried out using TC-7.0 buffer system. The results showed that the isozymes expression was highly tissue-specific in *S. esculenta*. Twelve isozymes (PGDH, GPI, MPI, IDHP, SOD, ME, AAT, DIA, MDH, LDH, G3PDH and PGM) and three kinds of tissues (eye, buccal bulb muscle and branchial heart) were selected for genetic analysis of *S. esculenta* population. Of the eighteen putative enzyme-coding loci examined, three loci were polymorphic, *i.e.*, *LDH-2**, *G3PDH-1** and *PGM**. The proportion of polymorphic loci was 0.166 7 ($P_{0.99}$) and 0.055 6 ($P_{0.95}$). The average values of observed and expected heterozygosity were 0.015 9 and 0.014 3, respectively. The average effective number of alleles was 1.020 1. Analysis of *S. esculenta* in the present study revealed low levels of genetic variability.

**Key words**: *Sepia esculenta*; isozyme; tissue-specificity; genetic structure
**CLC number**: Q959.216 **Document code**: A **Article ID**: 1005－8737－(2005)05－0549－07

The golden cuttlefish *Sepia esculenta* Hoyle, 1885 (Cephalopoda: Sepiidae) is widely distributed in the far east sea of Russia and the coast of China, Japan, Korea and Philippines. It is one of the most commercially important species of coastal fishes in China and Japan[1－2]. On account of its commercial importance, many aspects of biology of *S. esculenta* have been investigated: spawning season, spawning behaviour, migration, copulation, fecundity, egg, embryonic development, hatchling's body colour pattern and behaviour, resistance of hatchlings to low salinities, production of seedlings and aquaculture, locular index of cuttlebone, growth of shell, daily age markings on cuttlebone, and prey composition, and relationship between catch and tidal current[2－3]. But the aspects of its population biology are generally poorly documented. Considering the importance of this species for fisheries management, information of the populations is vital to the design and implementation of adequate management programs. The present study aimed at investigating its tissue-specificity and genetic structure of *S. esculenta*.

## 1 Materials and methods

### 1.1 Materials

Fourty-two individuals of *S. esculenta* in reproduction season were collected from the coast of Rizhao, Shandong Province, in June 2003. Body weight and mantle length were (579.11 ± 183.63) g and (148.26 ± 18.10) mm, respectively. The speci-

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mens obtained on the spot were immediately stored at −20 ℃ and transported to the laboratory in a frozen state, where six different kinds of tissues (eye, branchia, mantle muscle, buccal bulb muscle, branchial heart and liver) were taken from each individual and stored at −75 ℃ until required.

### 1.2 Methods

All samples to be analyzed for isozyme were homogenized with approximately equal volumes of frozen tissues and distilled water. After homogenation, the homogenate was centrifuged at 12 000 r/min for 12 min at 4 ℃ and the supernatant was absorbed by filter paper and was used for electrophoresis. Horizontal starch gel electrophoretic techniques and staining procedure followed the method of the Japan Fisheries Resource Conservation Association[4] and Pasteur et al.[5].

Electrophoresis was carried out at a constant current of 50 mA for 4 to 5 h. Nineteen isozymes were screened under TC-7.0 buffer system: phosphogluconate dehydrogenase (PGDH), glucose-6-phosphate isomerase (GPI), mannose-6-phosphate isomerase (MPI), isocitrate dehydrogenase (IDHP), superoxide dismutase (SOD), malic enzyme (ME), aspartate aminotransferase (AAT), diaphorase (DIA), malate dehydrogenase (MDH), lactate dehydrogenase (LDH), glycerol-3-phosphalate dehydrogenase (G3PDH), phosphoglucomutase (PGM), hexokinase (HK), leucyl naphtylaminopeptidase (LAP), alcohol dehydrogenase (ADH), glucose-6-phosphate dehydrogenase (G6PD), sorbitol dehydrogenase (SDH), esterase (EST) and alkaline phophatase (ALP). The names, numbers, and abbreviations of the enzymes followed Shaklee et al.[6] (Tab. 1). Six different kinds of tissues (eye, branchia, mantle muscle, buccal bulb muscle, branchial heart and liver) were surveyed in the preliminary experiment to find the isozymes and corresponding tissues suitable for population genetic research. The allele frequencies and observed heterozygosities for each locus were determined by direct census of the population data. A locus was considered to be polymorphic if the most common allele was equal or less than 0.99 or 0.95.

## 2 Results and analysis

### 2.1 Tissue-specificity

The results showed that the expression of isozymes was highly tissue-specific in *S. esculenta* (Tab. 2). Twelve enzymes (PGDH, MPI, GPI, ME, SOD, IDHP, DIA, PGM, G3PDH, MDH, AAT and LDH) showed various degrees of activity in the six different kinds of tissues (eye, branchia, mantle muscle, buccal bulb muscle, branchial heart and liver), while the others (HK, LAP, ADH, G6PD, SDH, EST and ALP) had no reaction. Of the tissues, buccal bulb muscle was the most suitable, most stable, and clearest for isozymes expression while liver was the worst. Eye and mantle muscle were next to buccal bulb muscle. Branchial heart was especially well for the reaction of Lactate dehydrogenase (LDH) and Superoxide dismutase (SOD). At the same time, malic enzyme (ME) was staining only in the eye tissue, and phosphogluconate dehydrogenate (PGDH) was staining in the eye and buccal bulb muscle tissues. The twelve enzymes and three kinds of corresponding tissues (eye, buccal bulb muscle and branchial heart) were selected for population genetic research.

### 2.2 Genetic structure

After initial screening, selected enzymes and tissues were used for genetic analysis of *S. esculenta* population. Photographs of electrophoretic patterns for these enzymes are shown in Fig. 1. Of the 12 enzymes assayed routinely, 3 loci (*G3PDH-1**, *PGM**, *LDH-2**) were polymorphic at $P_{0.99}$ level, while the others (*PGDH**, *GPI**, *MPI**, *IDHP**, *SOD**, *ME**, *AAT-1**, *AAT-2**, *DIA-1**, *DIA-2**, *MDH-1**, *MDH-2**, *MDH-3**, *LDH-1**, *G3PDH-2**) were monomorphic. There were two genotypes at the polymorphic loci, *i.e.*, **a/a* and **a/b* at locus *G3PDH-1** and locus *LDH-2**, **a/b* and **b/b* at locus *PGM** (Fig. 1, Tab. 3).

Genetic variability was estimated by the propor-

tion of polymorphic loci and average heterozygosity. The proportion of polymorphic loci was 0.166 7 ($P_{0.99}$) and 0.055 6 ($P_{0.95}$), respectively. The average observed and expected heterozygosity were 0.015 9 and 0.014 3, respectively. The average effective number of alleles was 1.020 1. For the polymorphic loci, the $\chi^2$ test for the Hardy-Weinberg equilibrium could not be performed because the expected values of the genotype frequency were smaller than five. Allele frequencies at the *LDH-2**, *G3PDH-1** and *PGM** loci deviated from expected Hardy-Weinberg proportions (Tab. 3). Genetic deviation index showed heterozygotes surplus at these loci (Tab. 3).

**Tab. 1 Names, abbreviations and E. C. numbers used for tissue-specificity test**

**表 1 用于组织特异性实验同工酶的名称、缩写和编号**

| Enzyme name | Enzyme abbreviation | E.C. number |
|---|---|---|
| Ghosphogluconate dehydrogenase | PGDH | 1.1.1.44 |
| Glucose-6-phosphate isomerase | GPI | 5.3.1.9 |
| Mannose-6-phosphate isomerase | MPI | 5.3.1.8 |
| Isocitrate dehydrogenase | IDHP | 1.1.1.42 |
| Superoxide dismutase | SOD | 1.15.1.1 |
| Malic enzyme | ME | 1.1.1.40 |
| Aspartate aminotransferase | AAT | 2.6.1.1 |
| Diaphorase | DIA | 1.6.2.2 |
| Malate dehydrogenate | MDH | 1.1.1.37 |
| Lactate dehydrogenase | LDH | 1.1.1.27 |
| Glycerol-3-phosphalate dehydrogenase | G3PDH | 1.1.1.8 |
| Phosphoglucomutase | PGM | 5.4.2.2 |
| Hexokinase | HK | 2.7.1.1 |
| Leucyl naphtylaminopeptidase | LAP | 3.4.11.1 |
| Alcohol dehydroenase | ADH | 1.1.1.1 |
| Glucose-6-phosphate dehydrogenase | G6PD | 1.1.1.49 |
| Sorbitol dehydrogenase | SDH | 1.1.1.14 |
| Esterase | EST | 3.1.1.1 |
| Alkaline phophatase | ALP | 3.1.3.1 |

**Tab. 2 Activities of twelve enzymes in six tissues of *Sepia esculenta***

**表 2 12 种同工酶在金乌贼 6 种组织中的表达状况**

| Enzyme | Branchia | Eye | Mantle muscle | Buccal bulb muscle | Liver | Branchial heart |
|---|---|---|---|---|---|---|
| PGDH | − | +* | − | + | − | − |
| GPI | + | + +* | + | + | + | + |
| MPI | + + | + + +* | + + | + + | − | + |
| IDHP | + + | + + | + + | + + +* | − | + |
| SOD | + | − | − | − | + + +* | |
| ME | − | +* | − | − | − | − |
| AAT | + + | + + | + + | + + +* | − | + + |
| DIA | + | + | + | + +* | + | + |
| MDH | + | + | + + | + + +* | − | + |
| LDH | + | + + | − | + | + | + + +* |
| G3PDH | + | + | + + | + + +* | − | − |
| PGM | + | + | + + | + + +* | − | + |

Note: "+ + +"—Strong; "+ +"—Moderate; "+"—Weak; "−"—No reaction; "*"—Tissue selected

注: "+ + +"—强; "+ +"—中; "+"—弱; "−"—无带; "*"—筛选出的组织

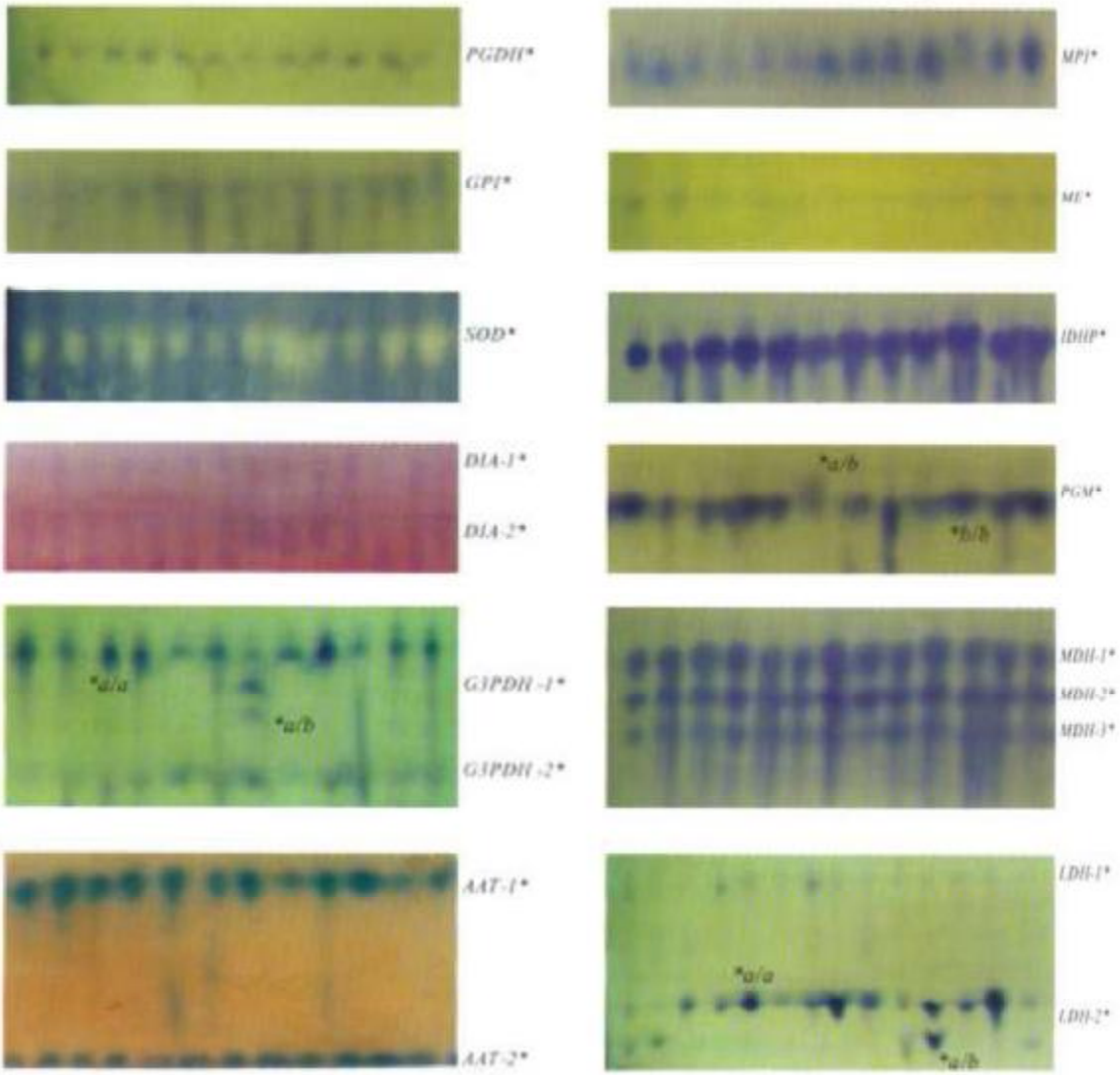


Fig. 1 Electrophoretic patterns of the twelve isozymes, in *Sepia esculenta*

图 1 金乌贼 12 种同工酶(PHDH, GPI, SOD, DIA, G3PDH, AAT, MPI, ME, IDHP, PGM, MDH and LDH)的电泳图谱

## 4 Discussion

The cephalopods belong to the highest class in the phylum Mollusca. Mantle muscle, liver, eye and buccal bulb muscle were thought to be highly developed organs in Mollusca[7]. The four kinds of tissues were used for isozyme analysis in *Sepiella maindroni*[8]. Mantle muscle and liver were used in the study of *Octopus ocellatus*[9]. Pérez-Losada et al.[10] suggested that mantle muscle rather than digestive gland should be used for routine electrophoretic studies in *Sepia* species obtained from commercial catches. Only buccal bulb muscle was selected for isozyme analysis in some species of cephalopods[4]. In this experiment, the buccal bulb muscle had better effect than mantle muscle, and mantle muscle got much better effect than liver. The buccal bulb muscle, eye and branchial heart were suitable for the selection for isozyme analysis of *S. esculenta*.

According to Nei[11], one of main objectives in

population genetics is to describe the amount of the genetic diversity in populations and then to study the maintenance of this diversity. Analysis of genetic variation in *S. esculenta* in present study reveals low levels of isozyme variability. The mean $H_o$(0.015 9) falls below the average of other cephalopods (0.03 ± 0.03)[12], marine mollusks (0.147)[13], molluscs and invertebrates (0.145 and 0.120, respectively)[14]. Low levels of heterozygosity have been found in other species of Cephalopoda, such as *Todarodes pacificus*, *Ommastrephes bartrami*, *Octopus vulgaris*, *Octopus minor*, *Octopus ocellatus* and *Callistoctopus magnocellatus*[4], *Pareledone turqueti*[15], *Loligo gahi* and *Illex argentinus*[16-17], *Sepioteuthis lessoniana*[18], *Sepia officinalis*, *Sepia orbignyana* and *Sepia elegans*[10, 19-21], *Sepia maindroni*[8], *O. ocellatus*[9]. Very low level of genetic variability in cephalopods is apparently a common character except *Berryteuthis magister* (0.131)[22]. Proportions of polymorphic loci of *S. esculenta* were 0.166 7 ($P_{0.99}$) and 0.055 6 ($P_{0.95}$) respectively, which was similar to the values reported for *O. ocellatus*[9] and *O. bartrami*[4], and below the average values of *S. officinalis*[19], *S. maindroni*[8], *T. pacificus*, *O. vulgaris*, *O. minor*, *O. ocellatus* and *C. magnocellatus*[4]. The average effective number of alleles was 1.020 1, which was below the *S. officinalis*, *S. orbignyana* and *S. elegans*[10, 19-21], *S. maindroni*[8] and *O. ocellatus*[9].

**Tab.3 Allele frequency, proportion of polymorphic loci, and average heterozygosity in *S. esculenta* population**
**表 3 金乌贼群体基因频率、多态位点比例和平均杂合度**

| Locus | Genotype | Observed numbers | Allele | Frequency | Genetic deviation index |
|---|---|---|---|---|---|
| *PGDH** | *a/a | 42 | *a | 1 | |
| *GPI** | *a/a | 42 | *a | 1 | |
| *MPI** | *a/a | 42 | *a | 1 | |
| *IDHP** | *a/a | 42 | *a | 1 | |
| *SOD** | *a/a | 42 | *a | 1 | |
| *ME** | *a/a | 42 | *a | 1 | |
| *AAT-1** | *a/a | 42 | *a | 1 | |
| *AAT-2** | *a/a | 42 | *a | 1 | |
| *DIA-1** | *a/a | 42 | *a | 1 | |
| *DIA-2** | *a/a | 42 | *a | 1 | |
| *MDH-1** | *a/a | 42 | *a | 1 | |
| *MDH-2** | *a/a | 42 | *a | 1 | |
| *MDH-3** | *a/a | 42 | *a | 1 | |
| *LDH-1** | *a/a | 42 | *a | 1 | |
| *LDH-2** | *a/a | 32 | *a | 0.881 | 0.1356 |
| | *a/b | 10 | *b | 0.119 | |
| *G3PDH-1** | *a/a | 41 | *a | 0.9881 | 0.0036 |
| | *a/b | 1 | *b | 0.0119 | |
| *G3PDH-2** | *a/a | 42 | *a | 1 | |
| *PGM** | *a/b | 1 | *a | 0.0119 | 0.0036 |
| | *b/b | 41 | *b | 0.9881 | |
| | | $P^*_{0.99}$ | | | 0.1667 |
| | | $P^*_{0.95}$ | | | 0.0556 |
| | | *Ho* | | | 0.0159 |
| | | *He* | | | 0.0143 |
| | | *Ae* | | | 1.0201 |

The results of the current study provided information on the genetic structure of *S. esculenta*. This information is needed to establish a genetic database, which can be used to make informed management decisions of this species for its important commercial position. To provide a better understanding of the genetic structure and future management on the golden cuttlefish, further study on more populations by many genetic methods is needed.

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