

不同罗氏沼虾种群形态差异的比较研究

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摘要:采用3种多元分析方法,对8个罗氏沼虾(*Macrobrachium rosenbergii*)种群(浙江养殖种群、缅甸引进种群及6个不同组合杂交种群)的12个形态比例参数进行比较研究。聚类分析和主成分分析法对雌、雄虾的分析结果表明,6个杂交种群的形态较为接近,而与养殖种群和引进种群的趋异程度逐渐增加。构建的5个雌体主成分中,方差贡献率分别为主成分(1)26.191%、主成分(2)16.036%、主成分(3)13.309%、主成分(4)10.769%、主成分(5)10.166%,累积贡献率为76.471%。相应地,雄体的方差贡献率分别为主成分(1)25.206%、主成分(2)20.277%、主成分(3)16.985%、主成分(4)13.222%、主成分(5)8.945%,累积贡献率为84.634%。判别分析结果显示,8种群之间的形态参数之间有很显著的差异($P < 0.01$);8种群雌体判别函数的判别准确率 P_1 为60.03%~100.00%, P_2 为33.30%~100%,综合判别率为92.1%;雄体判别准确率 P_1 为20.00%~100.00%, P_2 为20.00%~100%,综合判别率为86.4%。其中判别函数对养殖种群、缅甸引进种群和“反交正回交”3个种群的判别准确率较高。综上所述,养殖种群和引进种群在形态上已出现了明显的分化,并独立成为2个不同的种群;其他杂交种群由于同时综合了养殖种群和引进种群两者的相关性状,形态上没有呈现显著的差异($P > 0.05$)。[中国水产科学, 2006, 13(4): 530-535]

关键词:罗氏沼虾;种群形态差异;多元分析

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有关虾的遗传多样性研究,国内外已有很多报道。Garcia等^[1], Alcivar等^[2]分别分析了草虾(*Penaeus monodon*)和凡纳滨对虾(*Litopenaeus vannamei*)不同品系的遗传变异,并成功地筛选出了一些特异性标记。宋林生等^[3]用RAPD技术研究了日本对虾(*Penaeus japonica*)野生群体和养殖群体的遗传结构,确定了野生群体的多态位点(呈现多态现象的基因位点)比例和杂合度明显高于养殖群体^[3]。关于其他动物种群遗传多态性的研究还有很多,王冬群等^[4]、张全启等^[5]、李海燕等^[6]和方展强等^[7]分别对象山螯蜆(*Sinonovacula costaricta* Lamarck)、牙鲆(*Paralichthys olivaceus*)、中华绒螯蟹(*Eriocheir sinensis*)和鲈(*Siniperca chuatsi* Basilewsky)的野生种群与养殖种群遗传多态性进行了研究。

罗氏沼虾(*Macrobrachium rosenbergii*)为世界上的大型淡水虾类,其肉味鲜美、食性广、生长快,是1种优良的养殖品种,主要分布在东南亚国家。近10多年来中国开始引进,经过多代养殖和累代人工

繁殖,近年来其养殖种群的种质明显退化,主要表现为个体趋于小型化、性成熟提前和繁殖率下降等。但有关罗氏沼虾种群遗传多样性的研究,目前仅见甘西等^[8]、李明云等^[9]采用RAPD技术,对养殖群体和野生群体的遗传多样性进行比较,结果都显示养殖群体的多样性明显低于野生群体,并认为这与长期近亲繁殖和小群体间近交而导致种群退化有关,但有关罗氏沼虾种群形态差异的研究还几乎为空白。

遗传多样性是从遗传学的角度运用各种遗传学标记技术研究种群间及种群内的差异,对于动物的遗传育种有着十分重要的意义。与之相比,形态差异分析则能简单快速有效地反应不同种群之间的差异。本研究尝试用聚类分析、判别分析和主成分分析3种多元分析方法,对罗氏沼虾不同种群的形态比例参数进行比较,以期建立罗氏沼虾种群的形态鉴别方法,从而为进一步研究其种质遗传和苗种选育提供必要的参考。

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1 材料与方法

1.1 材料

实验材料取自浙江湖州省级南太湖淡水种业有限公司。每个实验组随机取性成熟虾(体长4 cm以上)30尾,8个种群遗传背景及代码(表1)。

1.2 形态参数测量

用游标卡尺测量每尾虾的全长、体长、头胸甲长、头胸甲宽、第二步足长、腹长、腹宽、尾长、尾宽,精确到0.1 mm;用天平称体质量和肝胰脏重,精确到0.01 g,具体方法参照文献[10]。

表1 实验用虾的代码和来源

Tab.1 Code and origin of experiment material

种群代码 Code of populations	编号 Trial No.	材料来源 Source
A	1	本地养殖种 The local cultivated population
B	2	引进子二代种群 The second offspring of Burma introduced population
B-+	3	"南太湖1号" "South Tailake One"
B++	4	引进种子一代♂×本地种♀ The first offspring of Burma introduced population♂×the local cultivated population♀
B+-	5	正交子一代♂×本地种♀ The first offspring of plus hybridization♂×the local cultivated population♀
B-	6	正交子一代♀×本地种♂ The first offspring of plus hybridization♀×the local cultivated population♂
B+	7	反交子一代♀×本地种♂ The first offspring of minus hybridization♀×the local cultivated population♂
B--	8	反交子一代♂×本地种♀ the first offspring of minus hybridization♂×the local cultivated population♀

注:正交子一代——引进种♀×本地种♂;反交子一代——引进种♂×本地种♀。

Note: The first offspring of plus hybridization: the Burma introduced population♀×the local cultivated population♂; the first offspring of minus hybridization: the Burma introduced population♂×the local cultivated population♀.

1.3 数据统计

实验数据用SPSS 11.5软件包处理;F检验法;聚类分析、主成分分析和判别分析3种多元分析方法,这3种方法是基于同时对多个对象进行考察,对所得数据的分析方法。

1.3.1 聚类分析 为消除虾体规格大小对参数值的影响,多元分析中采用以体长为基数或取2个形态参数比值。本研究选用12项形态特征比例参数,各参数代号为 A_1 :肝胰脏重/体质量(肝胰指数); A_2 :体质量/体长; A_3 :全长/体长; A_4 :头胸甲长/体长; A_5 :腹部长/体长; A_6 :尾扇长/体长; A_7 :第二步足长/体长; A_8 :头胸甲宽/体长; A_9 :腹部宽/体长; A_{10} :腹部宽/头胸甲宽; A_{11} :头胸甲宽/头胸甲长; A_{12} :腹部宽/腹部长。

1.3.2 主成分分析 所有数值处理同聚类分析方法,先进行校正处理后,从12个比例性状中通过计算机程序得出几个综合性指标,即互不关联的几个

主成分。主成分贡献率和累计贡献率的计算参照文献[11]。

1.3.3 判别分析 判别分析中判别准确率的计算参照李思发等[12]方法:

P_1 = 某罗氏沼虾种群判别正确的尾数/该种群实际尾数×100%,

P_2 = 某罗氏沼虾种群判别正确的尾数/判入该种群尾数×100%。

$$\text{综合判别率} = \frac{\sum_{i=1}^k A_i}{\sum_{i=1}^k B_i}$$

式中: A_i 为第*i*个种群中判别正确的尾数, B_i 为第*i*个种群中的实际尾数, k 为种群数。

2 结果

2.1 罗氏沼虾8种群的聚类分析

从图1和图2可见,雌、雄体8个种群的12个性状经聚类分析结果。无论是雄虾还是雌虾,都是B++、B+和B-这3个杂交种群之间的欧氏遗传

距离最短,表明它们之间的亲缘关系最近。雌虾依次归入的顺序为 B- -、A、B+ -、B,最后为 B- +;而雄虾的归入顺序依次为 B- +、B+ -、B- -、B,最后并入的是 A。雌、雄分析结果分歧较大的

是杂交组合 B- +,分析结果显示雌体的 B- +与其他种群亲缘关系最远,雄体分析的结果则与之相悖。值得一提的是,雌体 B- +与 B(缅甸引进种)的关系较雄体对应群体间的关系要更接近。

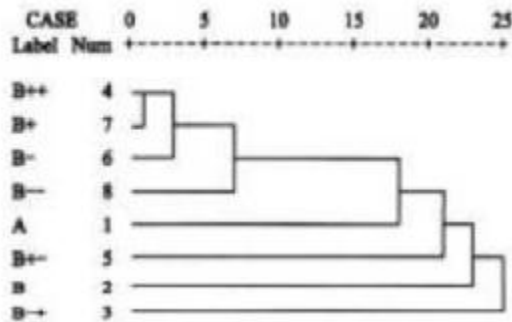


图1 罗氏沼虾雌体8种群形态聚类分析图

Fig.1 Clustering analysis on eight different populations of female *Macrobrachium rosenbergii*

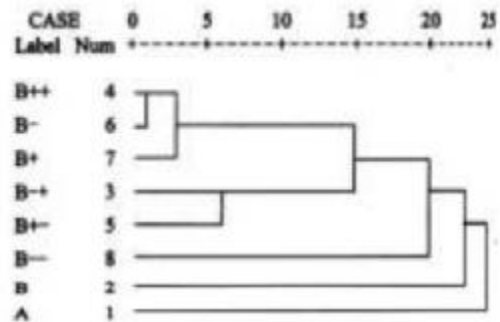


图2 罗氏沼虾雄体8种群形态聚类分析

Fig.2 Clustering analysis on eight different populations of male *Macrobrachium rosenbergii*

2.2 主成分分析

2.2.1 雌体 在主成分分析中,选用了方差贡献率较大的5个主成分,其贡献率分别为主成分(1)26.191%、主成分(2)16.036%、主成分(3)13.309%、主成分(4)10.769%、主成分(5)10.166%,累积贡献率为76.471%。5个主成分累积贡献率较高,包含了其总变异的绝大部分,表明可以用这5个相互独立的因子来概括不同种群雌虾间的形态差异。在第一主成分中, A_9 、 A_{10} 和 A_{12} 这3个变量影响最大。说明腹宽、腹长、头胸甲宽、体长这4个性状是决定这8个种群形态分类的主要依据。

2.2.2 雄体 在主成分分析中,选用了方差贡献率较大的5个主成分,其贡献率分别为主成分(1)25.206%、主成分(2)20.277%、主成分(3)16.985%、主成分(4)13.222%、主成分(5)8.945%,累积贡献率为84.634%。结果提示可以用这5个相互独立的因子来概括不同种群雄虾间的形态差异。和雌体的情况相同,在第一主成分中,也是 A_9 、 A_{10} 和 A_{12} 这3个变量影响最大。

图3和图4分别为雌、雄体8个种群的第1、2主成分的散布图,其中“主成分1”和“主成分2”为相对值(无量纲)。可以看出,8种群都有较多的不同程度的重叠区域,其中杂交种群重叠比例颇大,往往难以区分。本地养殖种和B- +这个杂交种群在雌体散布图上与其他种群重叠区域较少。

2.3 判别分析

对雌、雄体8种群的12个比例性状的判别F检验的结果表明,判别效果均较好($P < 0.01$),其中雌体的 A_3 和 A_{11} 性状差异不显著($P > 0.01$),判别准确率 P_1 为60.03%~100.00%,判别准确率 P_2 为33.30%~100%,综合判别率为92.1%。雄体的 A_5 、 A_{10} 和 A_{11} 性状差异不显著($P > 0.01$),判别准确率 P_1 为20.00%~100.00%,判别准确率 P_2 为20.00%~100%,综合判别率为86.4%。雌、雄判别分析结果都表明,A、B和B- + 3个种群的判别准确率较高,因此下面只列出这3个种群雌雄的判别方程式。

雌体

$$A: Y = 2143.433A_1 + 6.432A_2 + \dots + 4020.228A_{12} - 4515.830$$

$$B: Y = 1438.694A_1 + 7.049A_2 + \dots + 4587.086A_{12} - 2996.192$$

$$B- + : Y = 1350.945A_1 + 9.509A_2 + \dots + 5002.424A_{12} - 3130.721$$

雄体

$$A: Y = 1814.950A_1 - 1318.489A_2 + \dots + 246550.1A_{12} - 33495.0$$

$$B: Y = 1751.148A_1 - 1076.869A_2 + \dots + 243599.7A_{12} - 30374.7$$

$$B- + : Y = 1707.017A_1 - 1094.211A_2 + \dots + 242600.0A_{12} - 31941.1$$

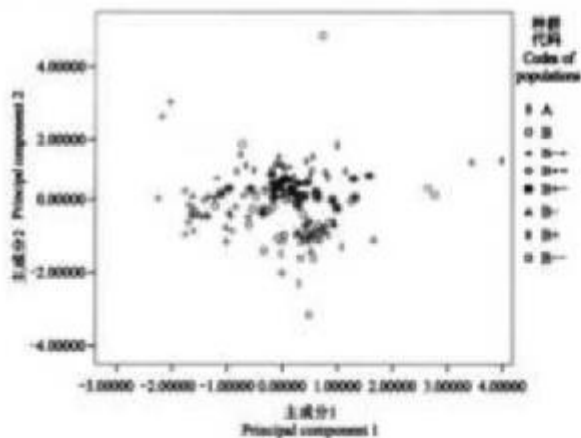


图3 8种群罗氏沼虾雌性第1,2主成分散布图
注:种群样品信息见表1。

Fig.3 Scatter diagram for first and second principal of eight female *Macrobrachium rosenbergii* populations

Note: Detailed information of samples in each population is in Table 1.

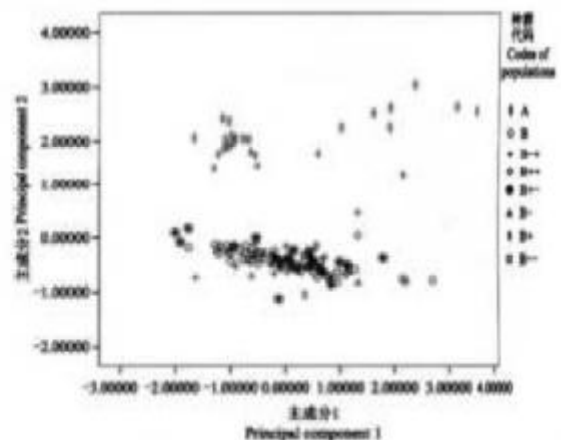


图4 8种群罗氏沼虾雄性第1,2主成分散布图
注:种群样品信息见表1。

Fig.4 Scatter diagram for first and second principal of eight male *Macrobrachium rosenbergii* populations

Note: Detailed information of samples in each population is in Table 1.

表2 8种群雌、雄体罗氏沼虾判别测试结果

Tab.2 Discriminative analysis results of eight female and male populations of *Macrobrachium rosenbergii*

种群代码 Code of populations	n		判别准确率/% Discriminant accuracy			
	♀	♂	$P_1(\frac{\bar{x}}{\bar{s}})$	$P_1(\frac{\bar{s}}{\bar{x}})$	$P_2(\frac{\bar{x}}{\bar{s}})$	$P_2(\frac{\bar{s}}{\bar{x}})$
A	30	26	100.00	89.66	100.00	100.00
B	24	30	80.00	100.00	100.00	100.00
B- +	29	29	96.67	96.67	100.00	100.00
B+ +	10	9	90.00	80.01	90.00	88.90
B+ -	23	25	79.31	73.33	100.00	88.00
B- -	6	9	19.98	70.02	33.30	77.80
B+ -	9	9	60.03	29.97	66.70	33.30
B- -	9	10	60.03	20.00	66.70	20.00
合计 Total	140	147	92.1	86.4		

3 讨论

采用聚类分析、主成分分析和判别分析这3种方法所得的结果相似,但各种方法从不同角度反映了不同种群(群体)间的形态学差异,因此各种方法之间不能相互替代。聚类分析可将不同种群进行初步归类,量化种群间的差异程度,分析种群间的相似程度;主成分分析则将多个形态比例性状综合成少数几个因子,从而得出不同种群差异的大小,并可根据不同种群的主成分值,找出对应种群(群体)在各主成分值上差异较大的参数;判别分析是种群鉴定的一种常用方法,通过建立判别函数来对种或种群

进行判别。多元分析和数值分类方法已被逐渐应用于育种和进化方面的研究,并在不同的生物类群的应用中获得了较好的效果,如三角帆蚌(*Hyriopsis cumingii* Lea)^[13],鳊属(*Parabramis pekinensis*)鱼类^[14],比目鱼(*Hippoglossus*)^[15],滨蛙(*Rana pleuraden*)和昭觉林蛙(*R. chaochiaoensis*)^[16]和哺乳动物山羊(*Capella*)^[17]等。

本研究采用聚类分析、判别分析和主成分分析3种多元分析方法^[18],分析了罗氏沼虾的11个形态参数和12个比例性状,结果表明,可以从形态上较好地地区分罗氏沼虾养殖种群、缅甸野生种群和不同组合的杂交种群。3种多元分析法的结果都显

示杂交种群之间(B-+除外)形态差异较小,而养殖种群(A)和引进种(B)与其他种群差异较大,与其他种群相差甚远,这符合遗传学的基因分离和自由组合规律;各杂交种群间雌体比雄体的差异相对较大,尤其是杂交种群(B-+)雌、雄间的差异尤为显著。主成分分析结果显示 A_9 、 A_{10} 和 A_{12} 这3个变量对其种群形态差异最大,表明腹宽、腹长、头胸甲宽、体长这4个性状是决定这8个种群形态分类的主要依据,同时也说明这4个性状是所分析性状中进化速度相对较快的性状。

养殖种群(A)和缅甸引进种(B)在形态学上也已经出现了明显的分歧,2个种群形态上的分化可能是由于养殖种群从引种后经过长时期的近亲之间、小群体的人工繁殖而导致的种群退化有关;而杂交种群(群体)间的亲缘关系较近,但也出现了一定程度的变异,杂交种群(B+、B-、B+-、B--、B-+、B+-)都由A(养殖种)和B(引进种)这2个种群以不同的方式杂交而产生的后代,在形态特征上它们继承了上述2个种群的遗传因子和综合表现性状,但一般来说,群体的表型决定于2对基因显隐性的相互作用^[19]。当以不同方式进行人为杂交的时候,显、隐性基因间的作用方式也不相同,从而导致不同杂交种群(B+、B-、B+-、B--、B-+、B+-)在形态上又出现了一定程度的变异。对雌、雄虾的分析结果显示,在形态上雌体比雄体的变异相对显著,特别是B-+种群,雄体与其他杂交种群的雄体几乎聚在一起,而雌体与其他杂交种群却产生了很大的分歧,这从文中的聚类分析和主成分分析图中均可看出。雌、雄虾差异可能是伴性遗传(sex-linkage)所导致的,由于B-+杂交组是由养殖种群(A)2次与引进种(B)雄体交配所得,其结果可能强化了引进种群雄体的优良性状在子代雌体上的表现,而雌体的表现则不明显,性染色体上基因的平衡不能由一代随机交配达到,而是要经过多代随机交配,且无其他因素干扰才能接近平衡,从而使同一基因的频率在两性达到一致^[20],因此,这个种群只有经过多代的随机交配之后,雄体才会表现出与雌体相同的杂种优势,这个结果符合一般的杂交育种的基本原理“远缘杂交”^[21],浙江养殖种雌体与缅甸引进种的雄体亲缘关系最远,它们杂交后代产生优良品种的可能性较大,值得在生产实践中加以关注。

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Morphological variations analysis among eight populations of cultivated and wild and hybridized in *Macrobrachium rosenbergii*

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Abstract: Much debate has surrounded the genetic diversity of shrimp. Garvca and Acivar have analyzed the genetic variation of *Litopenaeus vannamei* of different populations and filtrated differential markers successfully; Song Linsheng has investigated the genetic structure of the natural population and hatchery stock of *Penaeus Japonica*, and found that the mean proportion of polymorphic amplified fragments of natural population was higher than that of hatchery stock. The research about genetic diversity has also been made in other animal species such as *Sinonovacula const ricta* Lamarck, *Paralichthys olivaceus*, *Eriocheir sinensis* and *Siniperca chuatsi* Basilewsky. *Macrobrachium rosenbergii*, which is subjected to *Macrobrachium*, Palaemonidae, Crustacea, is a very important kind of freshwater edible shrimps in China and the economic value is prominent. In recent years, it has suffered degeneration in its resource, and its wild and cultivated stocks and their genetic diversity has been threatened to some extent due to overfishing and inbreeding. So it is the time to investigate the variation of *Macrobrachium rosenbergii* of different populations. Based on 12 morphological proportional characters of *Macrobrachium rosenbergii*, three multivariation analysis methods (cluster analysis, principal component analysis and discriminant analysis) were adopted to comparably investigate the morphological variation among eight populations (1 cultivated, 1 wild and 6 hybridized populations). The results of cluster analysis and principal component analysis indicated that the morphological characters were similar among the six hybridized populations. But quite different from that of cultivated and Burma introduced population. In the principal component analysis of the female populations, five principal components were constructed, the contributory ratio of the first principal component was 26.191%, the second, the third, the fourth and the fifth was 16.036%, 13.309%, 10.796%, and 10.166% respectively, the cumulative contributory ratio was 76.471%. Accordingly, for the male populations, five principal components were also constructed, their contributory ratios were 25.206%, 20.277%, 16.985%, 13.222% and 8.945% respectively, the cumulative contributory ratio was 84.634%. The result of discriminant analysis revealed that there were significant differences among most of the eight populations ($P < 0.01$). The identification accuracy of female was 60.03% – 100.00% (P1) and 33.30% – 100% (P2), the total discriminant accuracy was 92.1%. The identification accuracy of male was 20.00% – 100.00% (P1) and 20.00% – 100.00% (P2), the total discriminant accuracy was 86.4%. The results showed that there were distinct diversities between the cultivated population and Burma introduced population, which have formed two independent populations; but other six hybridized populations (with the exception of the population of B-+) did not appear distinct diversities, which was because these populations were the integrative offspring of the two populations mentioned above. [Journal of Fishery Sciences of China, 2006, 13(4): 530 – 535]

Key words: *Macrobrachium rosenbergii*; Morphological variation of populations; Multivariation analysis

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Cloning and tissue expression analysis of gonadotropin-releasing hormone receptor from the brain of Japanese flounder, *Paralichthys olivaceus*

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Abstract: A full-length cDNA, encoding a GnRH receptor (GnRH-R), has been obtained from the brain of Japanese flounder (*Paralichthys olivaceus*). This cDNA encodes a protein of 415 amino acid residues exhibiting the typical arrangement of the G-protein-coupled receptors with seven transmembrane domains. As already shown in other teleost fish, this protein possesses an intracellular carboxy-terminal tail, in contrast with its mammalian counterparts. In the case of Japanese flounder, this intracellular carboxy-terminal tail consists of 91 residues. Reverse transcription-polymerase chain reaction amplification products were found in the brain, the pituitary, the eyes and the ovary. [Journal of Fishery Sciences of China, 2006, 13(4): 536–546]

Key words: *Paralichthys olivaceus*; GnRH; GnRH receptor

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The decapeptide gonadotropin-releasing hormone (GnRH) has been proved to be the key information molecular in hypothalamus-pituitary-gonad axis and plays a central role in the neuroendocrine control of the reproductive process in all vertebrates, notably by stimulating synthesis and release of gonadotropins^[1]. However, GnRH may also deserve other functions such as neuromodulatory role in sexual behavior and transferring olfactory signals, immunomodulatory role and regulating cell proliferation role^[2–3]. The brain integrates the available information and transfers the signal to the gonadotropic cells either indirectly via the portal system (in tetrapods) or directly via axon endings (in the most teleosts)^[2]. Due to the important potential applications of GnRH in fish farming^[4] and to the fact that teleost fish have turned out to be of special interest to understand the mechanisms underlying the evolution of GnRH genes in vertebrates, research on GnRH system in teleost fish has focused increasing attention^[5].

With the progress of fish GnRH studies, a growing number of studies have been devoted to fish GnRH-R since the cloning of the first fish GnRH receptor (GnRH-R) in catfish^[6]. Since then, several GnRH-Rs have been cloned in various teleost species including goldfish^[7], rainbow trout^[1], African catfish^[8], medaka^[9], striped bass^[10], amberjack (AJ130876), African cichlid^[11], European sea bass (DLA419594), and Japanese eel^[12]. Fish GnRH receptors belong to the rhodopsin-like G protein-coupled receptors family (GPCRs), containing seven hydrophobic transmembrane domains connected by hydrophilic extracellular and intracellular loops. In contrast with the mammalian GnRH-Rs, the fish receptors contain an intracellular carboxy-terminal tail that is required for piscine GnRH-R function^[13].

In the present study, we report the presence of GnRH-R in Japanese flounder, *Paralichthys olivaceus*. We have isolated the cDNAs encoding GnRH-R and determined the nucleotide sequence, structural

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organization, evolution analysis and tissue distribution. This GnRH-R bears seven transmembrane domains characteristic of Gprotein-coupled receptor (GPCR) family and belongs to type I GnRH-R group.

1 Materials and methods

1.1 Animals

Mature female Japanese flounder (*Paralichthys olivaceus*) (700–800 g) were obtained from the Marine Fish Market in Qingdao City (Shandong Province, China) and kept under recycled water at natural temperature until use. The developmental stage of the fish was estimated by the time of the year and confirmed by the gonadosomatic index.

1.2 RNA Preparation and cDNA Synthesis

Total RNA was extracted from the brain of Japanese flounder using the TRIzol reagent (Invitro-

gen, USA) according to the manufacturer's protocol. Japanese flounder brain cDNA used for rapid amplification of cDNA ends was synthesized from 1 µg of total RNA using a SMART RACE cDNA Amplification Kit (BD Biosciences Clontech, Palo Alto, CA), according to the manufacturer's instructions. Briefly, a reverse transcription (RT) reaction was performed on 1 µg of total RNA using an 3'-RACE CDS primerA (3'-CDS; 5'-AAGCAGTGGTATCAACGCAGAGTAC(T)₃₀VN-3') for 3'-RACE and 5'-RACE CDS Primer (5'-CDS) for 5'-RACE. The cDNAs used as templates for the first partial cDNA amplification were reversely transcribed from 3 µg of denatured total RNA using 10 µM adaptor-dT primer and 200 units of M-MLV Reverse Transcriptase (Promega, Madison, WI) in a 25 µL reaction volume incubated at 42 °C for 1.5 h.

Tab.1 Primers for cDNA synthesis, amplification and RT-PCR of GnRH-R cDNAs

表1 cDNA合成、GnRH-R cDNA扩增和 RT-PCR 的引物

Name 名称	Primers sequence * 引物序列	Utilisation 应用
Adaptor-dT	5'-GACTCGAGTCGACGAATTCAA(T) ₁₇ -3'	cDNA synthesis
FGnRH-R1	5'-TGRTGACCTTYRTSGATGCC-3'	first-round PCR for partial cDNA amplification
RnRH-R2	5'-AGARGCCGTAGATGAYSGGGT-3'	first-round PCR for partial cDNA amplification
FGnRH-R2	5'-TGGAACRTCSACRGTVCAgTg-3'	nested PCR for partial cDNA amplification
RnRH-R1	5'-YAGGTAGTAKGGMGTOCAGCA-3'	Nested PCR for partial cDNA amplification
3'-FPouter	5'-CTCCAAACGACTGAAAAGCC-3'	3'-RACE of first round PCR
3'-FPinner	5'-GAACAACATCCCAGAGC-3'	3'-RACE of nested PCR
5'-RPouter	5'-TGAAGTCTCGGGATGAAOGATGGT-3'	5'-RACE of first round PCR
5'-RPinner	5'-GCCAGAGGGTTGAGGATAG-3'	5'-RACE of nested PCR
GnRH-R-F	5'-CTGCTGGACTCCATACTACC-3'	RT-PCR forward primer
GnRH-R-A	5'-ACTGAGAAGAAATGGCACTA-3'	RT-PCR reverse primer
Actin344	5'-AAAGCCAACAGGGAGAAG-3'	β-actin amplification
Actin847	5'-GATGCTGTTGTAGGTGGT-3'	β-actin amplification

Note: * Abbreviations for degenerate nucleotides: Y=C or T; R=G or A; M=A or C; S=C or G; K=G or T; V=G, A or C; N=G, A, T or C.

注: * 简并核酸的缩写, Y=C或T; R=G或A; M=A或C; S=C或G; K=G或T; V=G, A或C; N=G, A, T或C.

1.3 Molecular cloning of GnRH-R Precursor cDNA

A first partial cDNA was obtained after nest polymerase chain reaction (PCR) amplifications with degenerate primers designed from conserved regions of known GnRH-R genes. The first amplification was carried out using forward primer FGnRH-R1 and reverse primer RnRH-R2. A 553 bp of product was

then obtained by the nest PCR using forward primer FGnRH-R2 and reverse primer RnRH-R1.

The 3' extremity and 5' extremity of the GnRH-R cDNA was cloned by 3' RACE (rapid amplification of cDNA ends) or 5' RACE-PCR, using a 5'-3' RACE kit (BD Biosciences Clontech, Palo Alto, CA) according to the manufacturer's condi-

tions. A first amplification was carried out with 3' - FPouter for 3' - RACE or with 5' - RPouter for 5' - RACE in combination with UPM of the kit. The reaction conditions for the first amplification were as follows: 95 °C for 3 min; 30 cycles at 94 °C for 30 s, 68 °C for 30 s, 72 °C for 3 min; and 72 °C for 7 min. The 20-fold-diluted first amplification solutions served as templates for the nested PCRs with 3' - FPinner for 3' - RACE or with 5' - RPinner for 5' - RACE in combination with NUP of the kit. The conditions for nested PCR were as follows: 95 °C for 3 min; 30 cycles at 94 °C for 30 s, 68 °C for 30 s, 72 °C for 3 min; and 72 °C for 7 min. 3' - FPouter, 3' - FPinner, 5' - RPouter and 5' - RPinner were designed from the 553 bp cDNA. The amplified products were separated by electrophoresis in a 1.0% Tris-boric acid-EDTA (TBE) agarose gel and visualized with ethidium bromide staining and a UV transilluminator. DNA fragments were extracted using NucleoTrap Nucleic Acid purification kits (BD Biosciences Clontech, Palo Alto, CA). The PCR products were cloned into the *EcoR* V site of pMD 18-T vector (Takara Biotechnology (Dalian) Co., Ltd) and transformed into competent *E. coli* DH5a for sequencing.

1.4 Sequence analysis

Multiple alignments and phylogenetic analysis were carried out using ClustalW 1.81 and Mega3 programme by 1 050 bootstrap replicates respectively. This analysis was performed using the amino acid sequences of GnRH-Rs available from GenBank. The sequence sources, gene name abbreviations in this article and GenBank accession numbers are as follows: Greater amberjack (*Seriola dumerili*, SDR1, AJ130876), Japanese eel (*Anguilla japonica*, Jeel-R1, AB041327), European seabass (*Dicentrarchus labrax*, EscabassR1, AJ419594), striped seabass (*Morone saxatilis*, SeabassR2, AF218841), black seabream (*Acanthopagrus schlegelii*, Bsbream-R1, AY820276), Cichlid (*Oreochromis aureus* × *O. niloticus*, OAxON R1, Cichlid-R1: AY381299), Nile tilapia (*Oreochromis niloticus*, NtilapiaR2: AB111356), North African catfish (*Clarias gariepi-*

mus, NcatfishR1, AF329894), Japanese medaka (*Oryzias latipes*, JmedakaR1, AB057675), bullfrog (*Rana catesbeiana*, bullfrogR1, AF144063), chicken (*Gallus gallus*, ChickenR1, NM_204653; GnRH - R2, NM_001012609), leopard gecko (*Eublepharis macularius*, LgeckoR2, AB109032), Rio Cauca caecilian (*Typhlonectes natans*, RCCR1: AF174481), African clawed frog (*Xenopus laevis*, ACfrogR2: AF257320), pig (*Sus scrofa*, pigR2, NM_001001639), African green monkey (*Cercopithecus aethiops*, AGmonkey-R2, AF353988), and rhesus monkey (*Macaca mulatta*, RmonkeyR2, AF353987).

1.5 Tissue distribution analysis of Japanese flounder GnRH-R by RT-PCR

For the detection of GnRH-R mRNA, different tissues were collected and total RNA was prepared and reversely transcribed as described above with adaptor-dT primer. A 340 bp GnRH-R cDNA was amplified using the primers GnRH-R-F and GnRH-R-A. The reaction conditions were as follows: 94 °C for 5 min; 45 cycles at 94 °C for 40 s, 67 °C for 30 s, 72 °C for 40 s; and 72 °C for 5 min. The PCR products were analyzed by electrophoresis on a 1.0% TBE agarose gel.

2 Results

2.1 Cloning of a full-length cDNA for GnRH receptor of Japanese flounder

Using nest-PCR, a first cDNA of 553 bp was obtained at the second round PCR. Nucleic acid blast results on NCBI revealed that this 553 bp fragment showed about 90% identity with the GnRH-R genes of European sea bass (AJ419594), striped sea bass (AF218841) or *Seriola dumerili* (AJ130876). Translation blast results on NCBI showed that the predicted amino acid sequence of this fragment have about 92% identity with *S. dumerili* GnRH-R (CAB65407), 89% with *Astatotilapia burtoni* GnRH-R1 (AY705931) and 92% with *Dicentrarchus labrax* GnRH-R (CAD11992). This part was revealed to contain a part of the coding region spanning from transmembrane domain (TM) III to V.

The 3' extremity of Japanese flounder GnRH-R

The complete cDNA sequence of Japanese flounder GnRH-R was spliced together from the above three part cDNA and the total cDNA is about 1671 bp including 147 bp of 5' UTR, 1248 bp of entire ORF and 276 bp of 3' UTR (Fig. 1). The sequence of this cDNA has been deposited in the GenBank (accession number DQ011872).

7个跨膜结构域以下划线和罗马数字表明。AATAAA 为 *poly(A)* 信号。基因登录号为 DQ011872。

2.2 Sequence analysis and comparison of GnRH-R with other GnRH-Rs

The nucleotide sequence and the deduced amino acid (aa) sequence of Japanese flounder GnRH-R are shown on Fig.1. The ORF codes 415 amino acid residues. Hydrophobicity analysis by the method of Kyte and Doolittle (Fig.2) and the transmembrane domain prediction of the deduced aa sequence showed that it bears seven transmembrane domains which is characteristic of Gprotein-coupled receptor. The primary structure of the GnRH-R contains an extracellular N-terminal tail of 44 aa, seven transmembrane

segments typical of a GPCR, three extracellular loops, three intracellular loops and an intracellular C-terminal tail of 91 aa (Fig.1 and Fig.3). Similar to the other teleost GnRH-Rs, but in contrast to mammalian GnRH-Rs, Japanese flounder GnRH-R possesses a C-terminal tail. It shows highest homology to sequences of other GnRH receptors, followed by sequences from corazonin receptor, arginine vasopressin receptor and mesotocin receptor. In particular, Japanese flounder GnRH-R cDNA we found is most similar to the GnRH-R sequence reported in *Seriola dumerili* (85% identity).

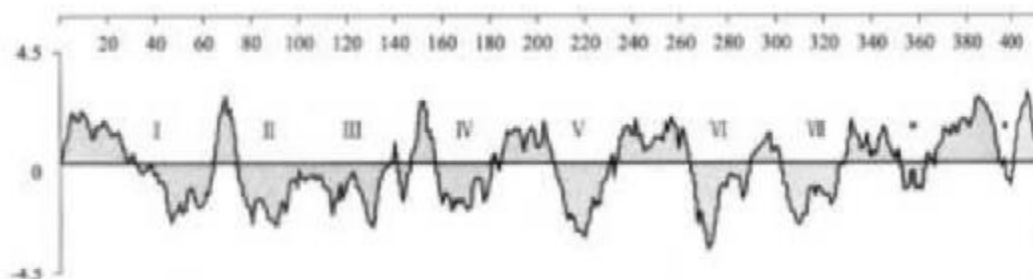


Fig.2 Hydrophathy plot of the Japanese flounder GnRH-R, using the method of kyte and doolittle

Note: The x-axis gives the amino acid residue number, and the y-axis the hydrophilicity index. The plot yields seven transmembrane domains (indicated by I - VII) that align with the transmembrane regions of other G protein-coupled receptors (Fig.3).

图2 牙鲆 GnRH 受体的 Kyte 和 Doolittle 亲水性图

注: x 轴代表氨基酸序列, y 轴代表亲水性参数。图中显示了 7 个潜在的 G 蛋白偶联受体多序列比对(图 3)的结果一致的跨膜结构域(以 I 至 VII 标注)。

Alignment of the predicted Japanese flounder GnRH-R amino acid sequence with the corresponding sequences described in such as catfish, sea bass, bullfrog, pig and so on was performed using Clustal W (Fig.3). The alignment showed that the GnRH-R amino acid sequences bear many conservative regions, especially at the hydrophobic region and transmembrane regions (Fig.2 and Fig.3).

A Neighbor-Joining (NJ) phylogenetic tree was drawn with Mega3 program (Fig.4). These analyses showed that the Japanese flounder GnRH-R is closely related to the other fish GnRH-R and particularly to the Greater amberjack *Seriola dumerili* GnRH-R1 (85% identity). However it shares only 49.6% identity with the bullfrog GnRH-R1 (amphibian), 39.2% with Rmonkey GnRH-R2 (mammalian), and 47.9% with chicken GnRH-R1 (bird). The tree also shows that the

Japanese flounder GnRH-R is more homologous to teleost GnRH-R1s than GnRH-R2s and GnRH-Rs of other species such as amphibian, bird, and mammalian. So it may be deduced that the cloned Japanese flounder GnRH-R is type I gonadotropin-releasing hormone receptor.

2.3 Tissue distribution of the GnRH-R in Japanese flounder

The location of GnRH-R could suggest its physiological roles. RT-PCR revealed that the GnRH-R mRNA has widespread distribution in Japanese flounder, GnRH-R mRNA is strongly expressed in brain, testes, kidney, eyes, and ovary (Fig.5). A weaker signal was also obtained in liver and testicle. The expression of this mRNA in both eyes and pituitary strongly indicates that this receptor is probably activated by multiple forms of GnRH *in vivo*.

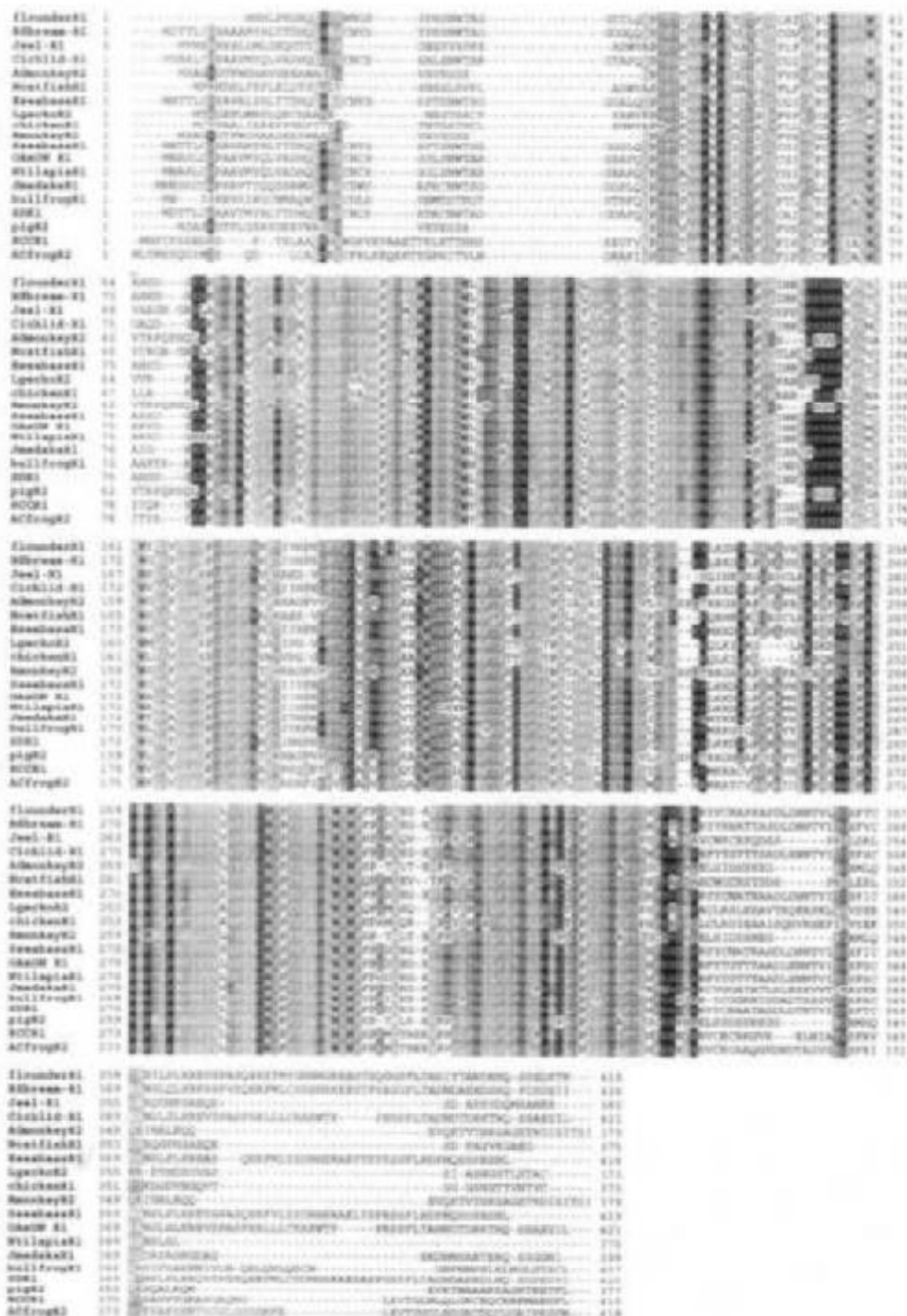


Fig.3 Alignment of GnRH-R aa sequences

Note: Putative transmembrane regions (TM I - TM V) are indicated by horizontal bars above the alignment. Sequences of identity or similarity are shaded with different color.

图 3 GnRH 受体氨基酸序列的多序列比对

注:潜在的跨膜结构域(TM I - TM V)用序列上的水平线标注,同源性相似的序列以不同的颜色阴影来表示。

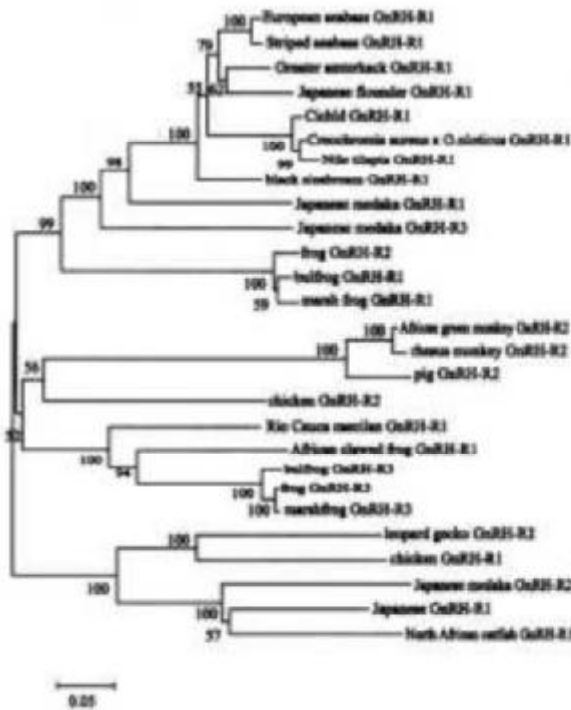


Fig. 4 Amino acid sequence phylogenetic analysis of the GnRH-Rs

图4 GnRH受体氨基酸序列的系统发育分析

3 Discussion

In the present study, we have cloned a cDNA of GnRH-R from Japanese flounder brain and studied the localization of the corresponding mRNA in different tissues by RT-PCR. In this cDNA, a methionine codon is found at the position 31, 148 and the stop codons are at the same position. However, in-frame ATG, at 148, could give rise to a protein with putative transmembrane domain typical of G-protein coupling receptor family. Multiple transcription start sites have already been reported in many genes, including GnRH-R as for example in human^[14], sheep^[15] or rainbow trout^[1].

Phylogenetic analysis reveal that Japanese flounder GnRH-R is more closely related to the greater amberjack GnRH-R1 (85% identity). Phylogenetic analysis of these teleost GnRH-R indicates that the presence of two main types, termed type I and type II GnRH-R, and examination of the aa sequences of type I and type II receptors revealed the presence of divergent motifs: all type I receptors have a C/GAFVT

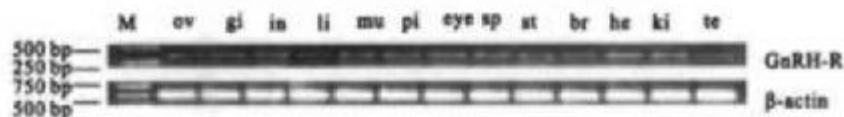


Fig. 5 Distribution of GnRH-R in Japanese flounder tissue assessed using RT-PCR

Note: br - brain; gi - gills; he - heart; in - intestine; ki - kidney; li - liver; mu - muscle; ov - ovary; pi - pituitary; sp - spleen; at - stomach; te - testicle.

图5 RT-PCR检测GnRH受体在牙鲆组织中的分布

注: br - 脑; gi - 鳃; he - 心脏; in - 肠; ki - 肾脏; li - 肝脏; mu - 肌肉; ov - 卵巢; pi - 垂体; sp - 脾脏; at - 胃; te - 睾丸。

motif in TM III whereas type II exhibit a SAFIL type motif; all type I receptors have a DLEGKVSHSLTH like sequence at the beginning of TM VII, while type II have a VTPEY motif at this site^[5]. In the Japanese flounder GnRH-R aa sequences, there are a CAFVT motif in TM III and a DLEGKVSHSLTH motif at the beginning of TM VII, which are the characteristics of the type I receptor. The above fact is accordant to the phylogenetic analysis that the GnRH-R obtained from Japanese flounder belongs to type I GnRH-R. The present

studies have the evidence that there are two or more subtypes in the single teleost fish, mammals or amphibians^[5,16-17]. whether a second potential GnRH-R cDNA exists in the Japanese flounder is required to be conformed.

The alignment of the primary amino acids sequence of Japanese flounder GnRH-R with the other species shows that a number of key features are conserved. Japanese flounder GnRH-R shows three main functional domains that include N-terminal extracellular domain (44 aa), a large transmembrane domain

(280 aa), and a C-terminal cytoplasmic domain (91 aa). The transmembrane domain is constituted of seven highly conserved transmembrane alpha helices that are required to anchor the receptor to the cell membrane.

This teleost fish GnRH-R bears an Asp⁸⁵ residue in transmembrane domain II that is conserved among members of fish GnRH-Rs, in the contrast an Asn residue in mammalian GnRH-Rs receptors, is required for piscine receptor function^[13]. In TM VII, GnRH-R bears an Asp³¹⁷ conserved among mammalian and fish, but this Asp is not required for GnRH-R functions^[51].

The regions between the alpha helices form intracellular or extracellular loops and were proved to be involved in signal transduction and ligand selectivity. At the cytoplasmic border of the TM III, there is a motif DRQSAI (DRXXXI/V) that may be involved in Gprotein-coupled signal transduction and GnRH ligand-induced receptor activation^[18-19]. Another conserved domain is the NPXXY or DPXXY motif located in TM VI (DPVIY in the Japanese flounder GnRH-R). This motif has been shown to be involved in the internalization of some GPCR including the GnRH-Rs and at the same time this motif especially the Tyr in this motif may be critical for receptor activation and signal transduction^[20]. In the third IC, there is a residue Ala (Ala²⁵⁸ in the Japanese flounder) conserved among fish GnRH-R which was determined to be also critical for Gprotein-coupling and receptor internalization^[21].

In the mammalian or non-mammalian GnRH-R, a disulfide bridge formed by two cysteines between the first and second ECs is conserved that is necessary for the correct folding of the receptors^[22]. The Japanese flounder GnRH-R, like other GnRH-Rs, presents a putative disulfide bridge between two cysteines (Cys¹¹² and Cys¹⁹⁰) sited in the first and second EC loop respectively. The complete absence of this bridge affected the structural integrity of the receptors and decrease its selectivity for GnRH^[23].

The N-terminal domain is less conserved, but contains glycosylation sites which may be required for

GnRH-R expression, trafficking of the GnRH-Rs to the cell surface or its stability^[22]. Introduction of the additional mouse receptor glycosylation site into the human receptor increased receptor number but not affected receptor-binding affinity or ligand selectivity^[24-25]. The Japanese flounder GnRH-R contains three potential glycosylation sites at its N-terminus (N¹¹SSW, N¹⁵GSS, N²²WTA), one in the first EC loop (N¹⁰⁰ITV) and one in the second EC loop (N¹⁷⁸VTI), which has more potential glycosylation sites than the mammalian counterparts. Further studies should be carried out to determine whether and how the glycosylation site numbers affect the level of expression, degradation rates or affinity of the piscine GnRH receptors.

GnRH mediates a series of signal transduction pathways: binding of GnRH to the receptor activates phospholipase C; phospholipase C hydrolyzes phosphatidylinositol 4,5-bisphosphate to generate both inositol 1, 4, 5-triphosphate (IP3) and diacylglycerol (DAG); IP3 is able to mobilize Ca²⁺ influ from intracellular stores and activate protein kinase C (PKC); and then the cAMP-adenylate cyclase system is involved in the relay of GnRH toward the release of LH^[22]. GnRH elicits GTP loading on both G_s and G_{q/11} and increased the levels of intracellular cAMP and GnRH-Rs may couple to the G_{q/11}-linked PKC, G_s-linking PKA or the both signaling pathway^[26-27]. In the first IC of the Japanese flounder GnRH-R, a motif (⁶⁹KRKSH⁷⁴) identical to GnRH-R III (⁸⁰KRKSH⁸⁴) is present which is a G_s-recognition motif (BBXXB, where B is a basic amino acid and X is any amino acid). It may be deduced that this motif is relevant with cAMP production as cGnRH-II increased stbGnRH-R activity in COS-7 cells via a cAMP-PKA pathway^[28].

Similar to other nonmammalian GnRH-Rs, the Japanese flounder receptor contains a C-terminal intracellular tail. This intracellular tail is required for piscine GnRH-R functions because the truncation of this domain produces the loss of both the ability for agonist binding and GnRH-stimulated cAMP produc-

tion^[29]. Likewise, addition of cGnRH-R C-terminal tail to the rat GnRH-R affects its expression, regulation, and the differential coupling with G proteins, producing a rapid desensitization of inositol phosphate production as well as increased internalization rate^[13,30-31]. In the third IC loop, the Japanese flounder GnRH-R contains two PKC phosphorylation site (233 SKR²³⁵ and 262 TLK²⁶⁴). In the C-terminal tail, it also has one PKC phosphorylation site (402 TAR⁴⁰⁴). Src homology 3 (SH3) binding domain (PxxP sequence)^[32] in the carboxy-terminal tail, which could potentially convey the possibility of coupling to mitogen-activated protein kinases (MAPKs)^[33], is also present in the Japanese flounder GnRH-R. In tilapia GnRH regulates GPα and LHβ transcription via PKC and PKA, both converging at the MAPK level, whereas the regulation of FSHβ transcription by GnRH is carried out via PKA-dependent, but PKC and MAPK-independent pathways^[34].

In conclusion, a GnRH-R cDNA of 1 671 bp has been cloned from Japanese flounder trout and may encode a protein with a 44 aa extracellular tail and an 191 aa intracellular tail, in contrast with its mammalian counterparts. This intracellular tail is required for piscine GnRH-R function^[13] and has been involved in the desensitization of a chimeric receptor by decreasing the rate of internalization^[30]. The corresponding mRNAs are expressed in retina, brain, pituitary, and ovaries. In the brain, the highest expression is clearly associated with a mesencephalic structure, the nucleus lateralis valvulae, whose precise function is unknown but may concern the processing of sensory or motor information.

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牙鲆脑中促性腺激素释放激素受体的克隆和鉴定

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摘要: GnRH(gonadotropin-releasing hormone)是下丘脑-垂体-性腺轴中重要的十肽信息分子,在所有的脊椎动物的生殖神经内分泌调控中具有重要的作用。GnRH成熟的十肽沿着轴突被运送到下丘脑正中隆起(median eminence)末端,然后进入下丘脑垂体门脉循环(hypothalamo-hypophyseal portal circulation)(四足动物)或直接通过轴突末梢(大多数硬骨鱼)把信号传递给刺激性腺的细胞,与特异性受体结合,刺激促性腺激素(GnHs, gonadotropins)的合成与释放,参与脊椎动物繁殖的起始和维持正常生殖功能。伴随着 GnRH 的研究,研究者对鱼类 GnRH 受体的研究也越来越感兴趣,牙鲆(*Paralichthys olivaceus*)是中国重要的海水养殖鱼类,本研究从牙鲆脑组织中克隆得到全长的 Type I GnRH 受体,并对其组织特异性表达作了分析,为牙鲆的神经生殖内分泌研究奠定了一定的基础。