Identification and characterization of gene involved in the growth rate of the Starry flounder, *Platichthys stellatus*

장요순

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한국해양과학기술원

Muscle Development and Growth in Fish

Objectives of the muscle research

KIOST

Studies of skeletal muscle

- ❖ Understanding mechanisms specifying cell fates
- ❖ Exploring the control of cell proliferation and differentiation
- ❖ Transcriptional regulators of the myogenic pathway
- ❖ Regulation of skeletal muscle growth

Studies of skeletal muscle growth

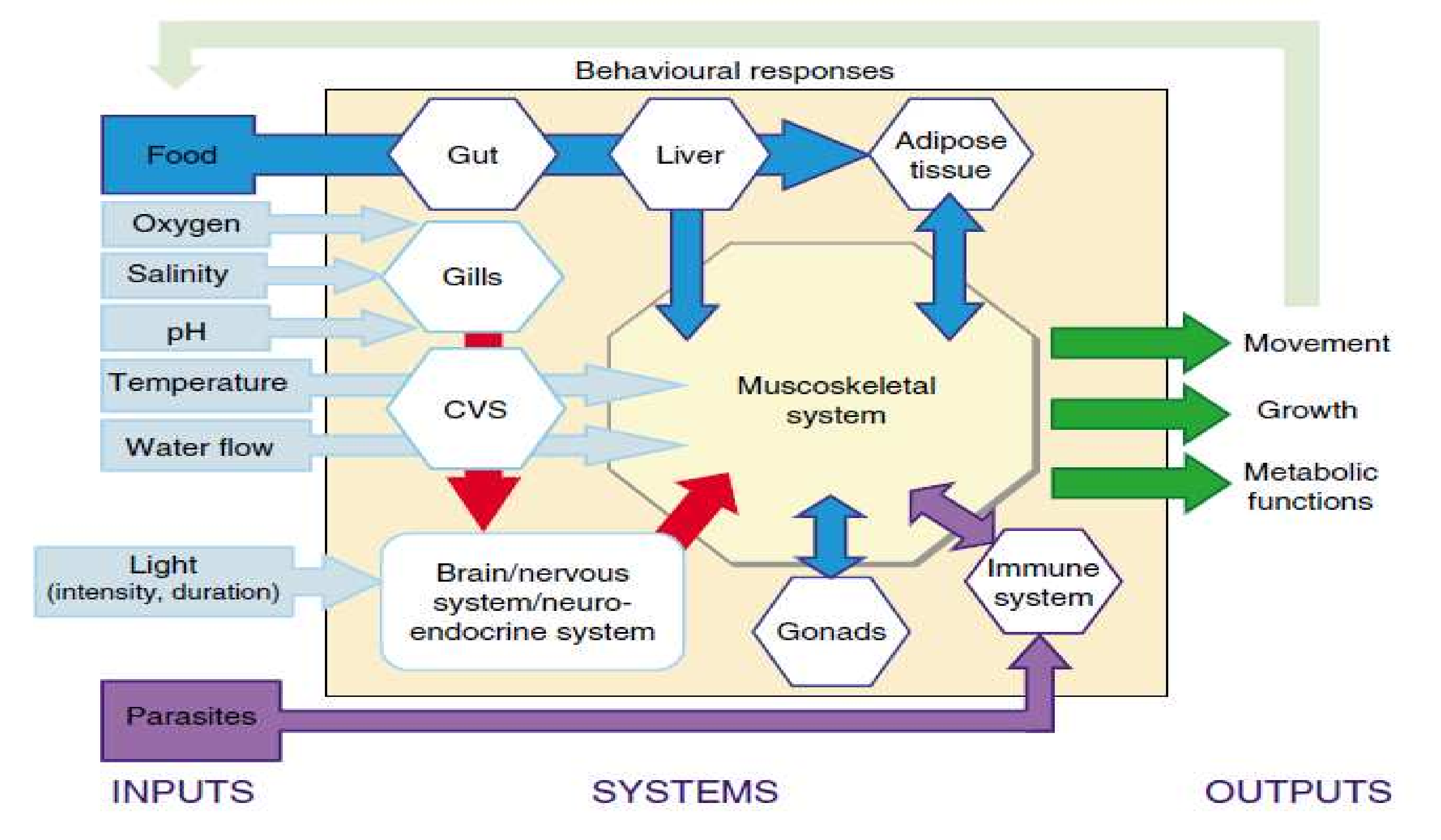
- *The skeletal muscle constitutes the edible part of the fish*

- ❖ Important for the development of fish farming
- ❖ Selective breeding program
- ❖ Dramatically increasing muscle growth rate

Myogenesis in teleost fish

Affect the functional outputs of skeletal muscle

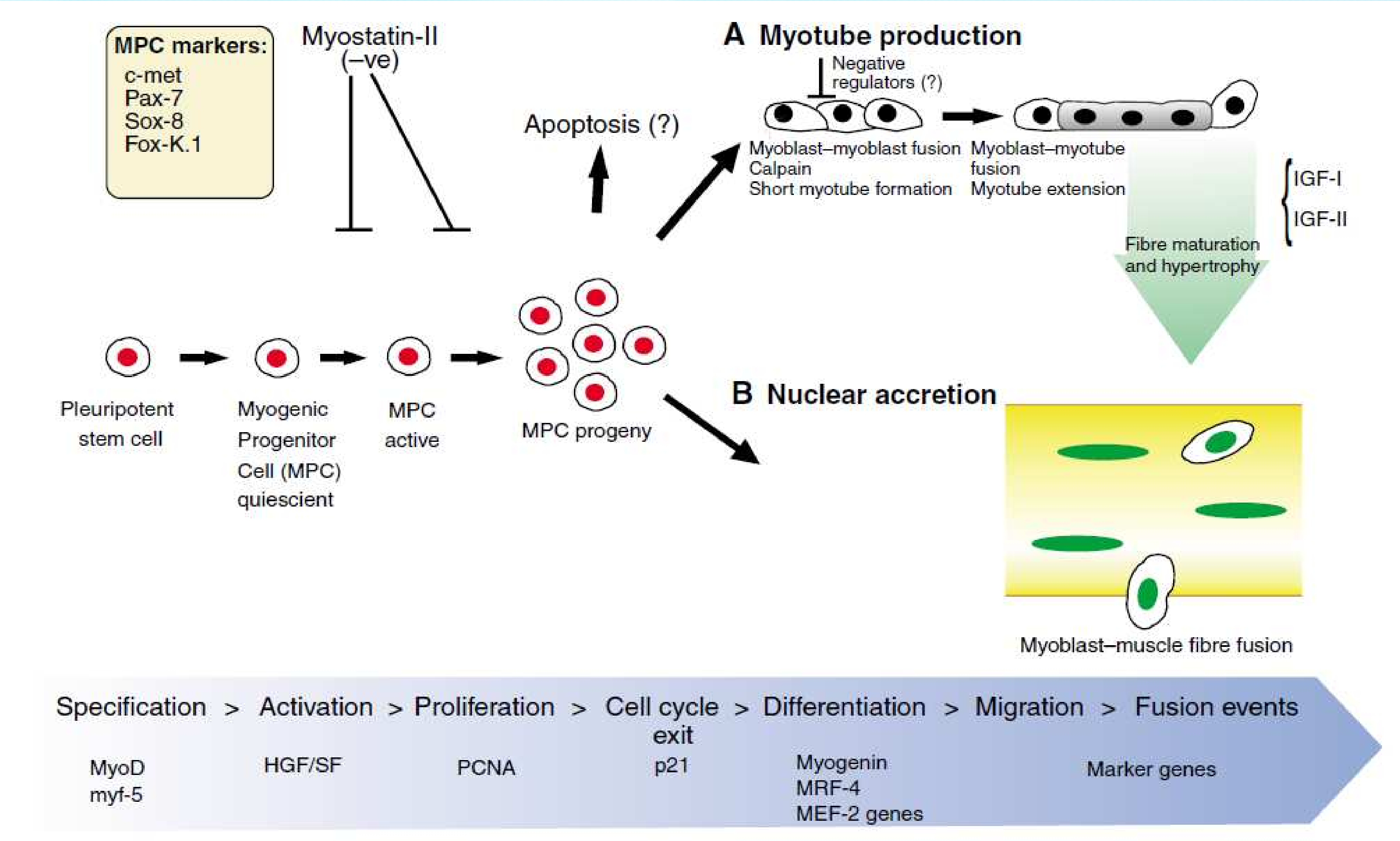
Ian A. Johnston, 2006



Myogenesis in teleost fish

Main event of myogenesis in teleost skeletal muscle

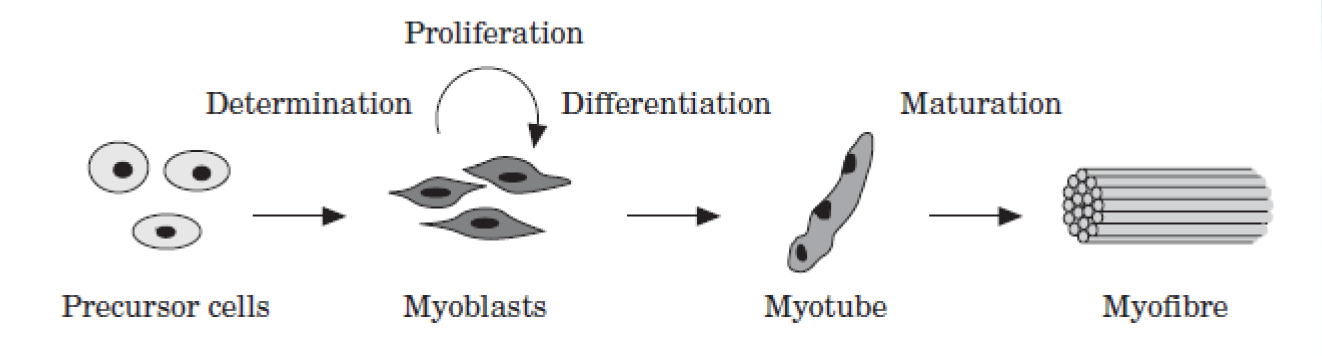
Ian A. Johnston, 2006



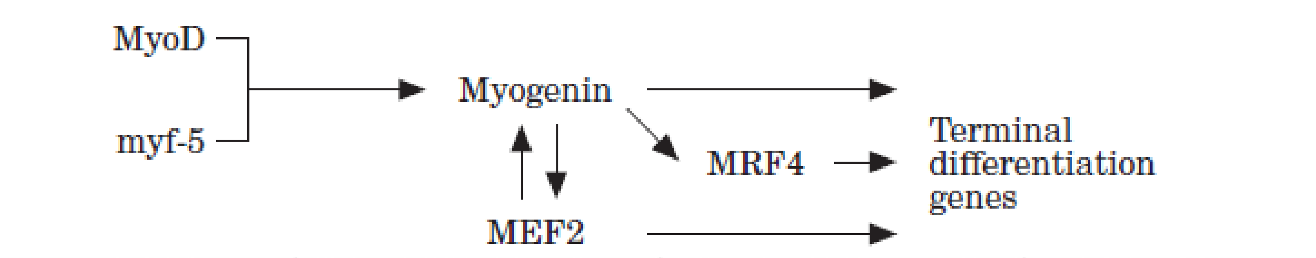
Myogenesis in teleost fish

Myogenic lineage determination and differentiation

S. Watabe, 1999



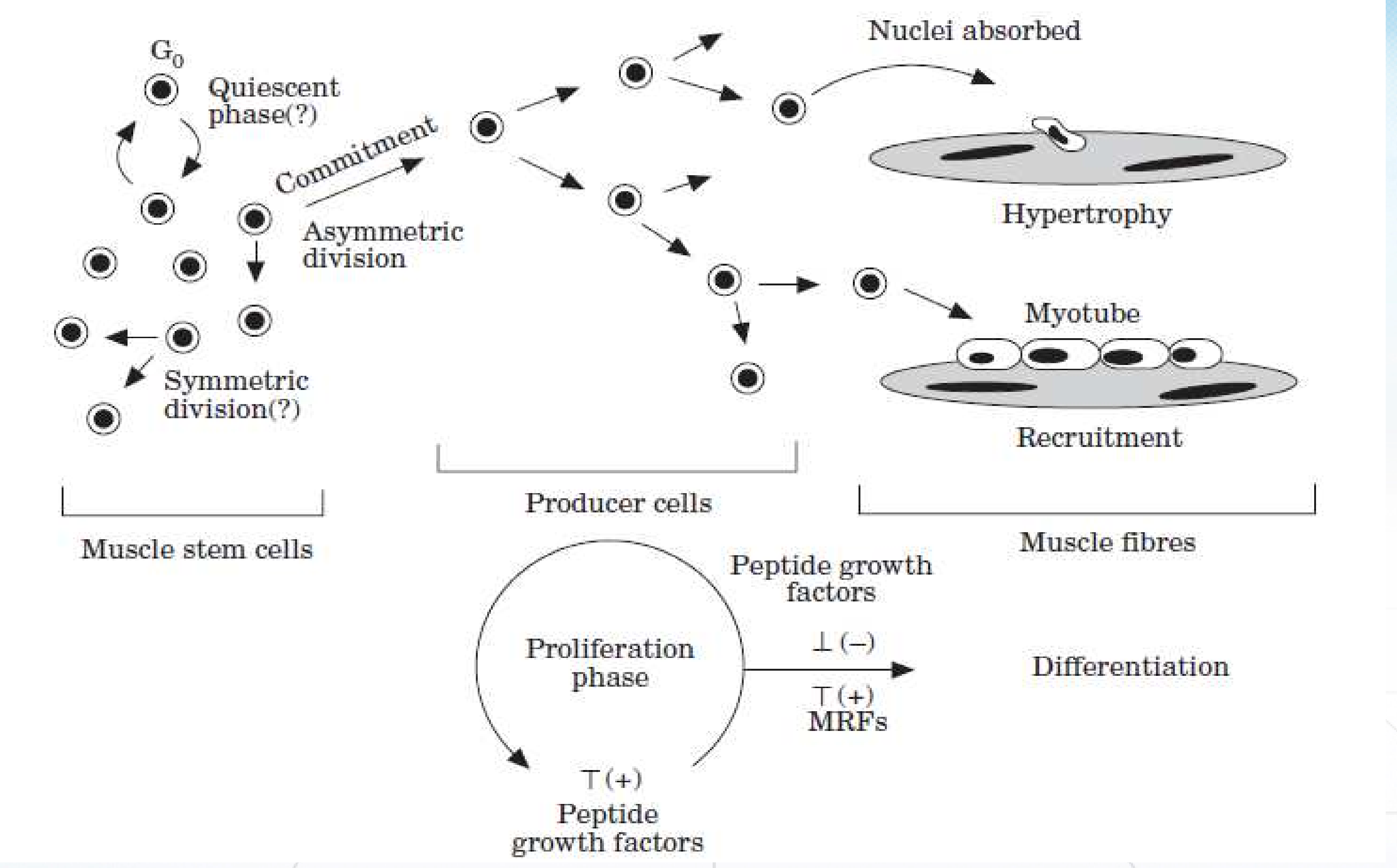
MEF2 family transcription factors together with muscle-specific genes



Myogenesis in teleost fish

A model for the cellular basis of muscle growth in fish

Ian A. Johnston, 2006



Fish Muscle Research Group (FMRG)

Introduction of FMRG

- **The University of St Andrews**
  - **The School of Biology**
  - **The Scottish Oceans Institute**

- **Topic**
  - **Teleost Fish**
    - ✓ **Contractile proteins**
    - ✓ **Developmental plasticity**
    - ✓ **Flesh quality traits**
    - ✓ **Gene characterization, discovery & expression**
    - ✓ **Muscle fiber types, Muscle growth, Muscle metabolism**

Current Research of FMRG

Molecular biomarkers of muscle growth

- Gene-Nutrient interactions
  - Atlantic salmon
  - Fast and slow growing strains

Functional genomics of muscle growth

- To identify and elucidate the function of novel genes
  - The genes controlling myotome development
- To investigate how temperature and stress
  - At the embryo and larval stage
  - The growth and differentiation of muscle and skeleton

Influence of selection for body size on muscle growth in the zebrafish

- ❖ **Investigate the relationship**
  - ➢ **Energy intake, food composition and quality**
  - ➢ **Somatic and muscle growth**

- ❖ **Understand the genetic basis of difference**
  - ➢ **Growth rates and its physiological implications**

- ❖ **Investigate developmental plasticity**
  - ➢ **Related to maternal and embryonic environment**

Maternal mRNA as molecular marker

❖ For egg quality in Atlantic halibut

- Knowledge of developmental mechanisms in farmed fish species
- Development of halibut farming through using biomarkers of egg quality

☞ *The function and phylogenetics of potential unknown marker genes will be studied*

Regulation of skeletal muscle growth in fish

KIOST

Characterization of nutritionally regulated genes

Physiol genomics 2010, 42A:114 -130

❖ **Nutritionally responsive candidate genes**

- **An independent fasting-refeeding experiment**
- **Fish with zero growth rates to fish growing rapidly**

Expression profile for growth-related genes

Aquaculture 2010, 307: 111-122

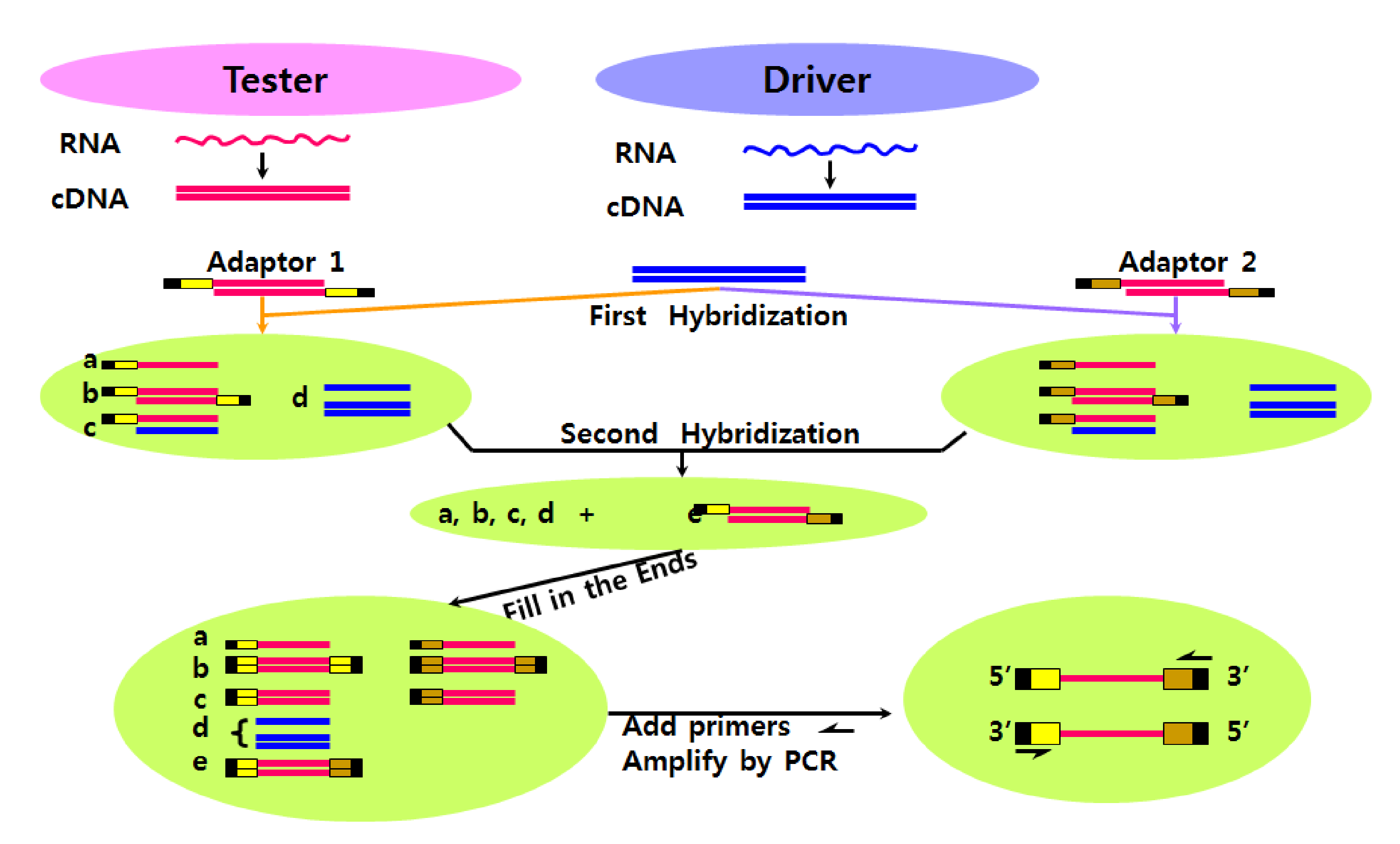
❖ **Genes related to muscle growth, metabolism, immunology and energy regulation**

- **In coho salmon (*Oncorhynchus kisutch*)**
- **Wild-type / domesticated (selected for growth) / growth hormone-transgenic (fed to satiation) / growth hormone-transgenic (restricted rations)**

Identification of genetically regulated muscle growth-related genes

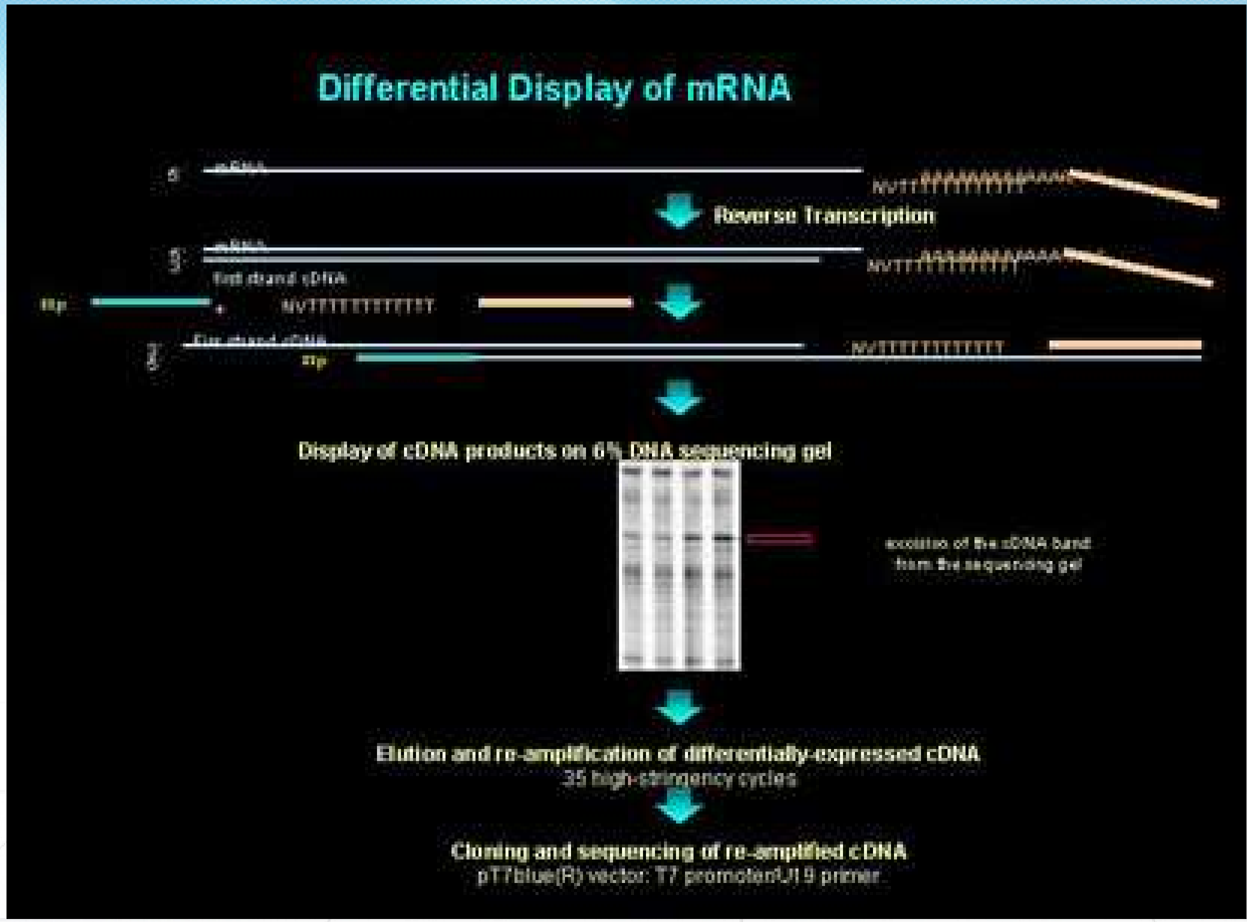
General method for differential screening

Subtractive hybridization



General method for differential screening

Differentially Display of mRNA



General method for differential screening

ACP- based GeneFishing™ PCR

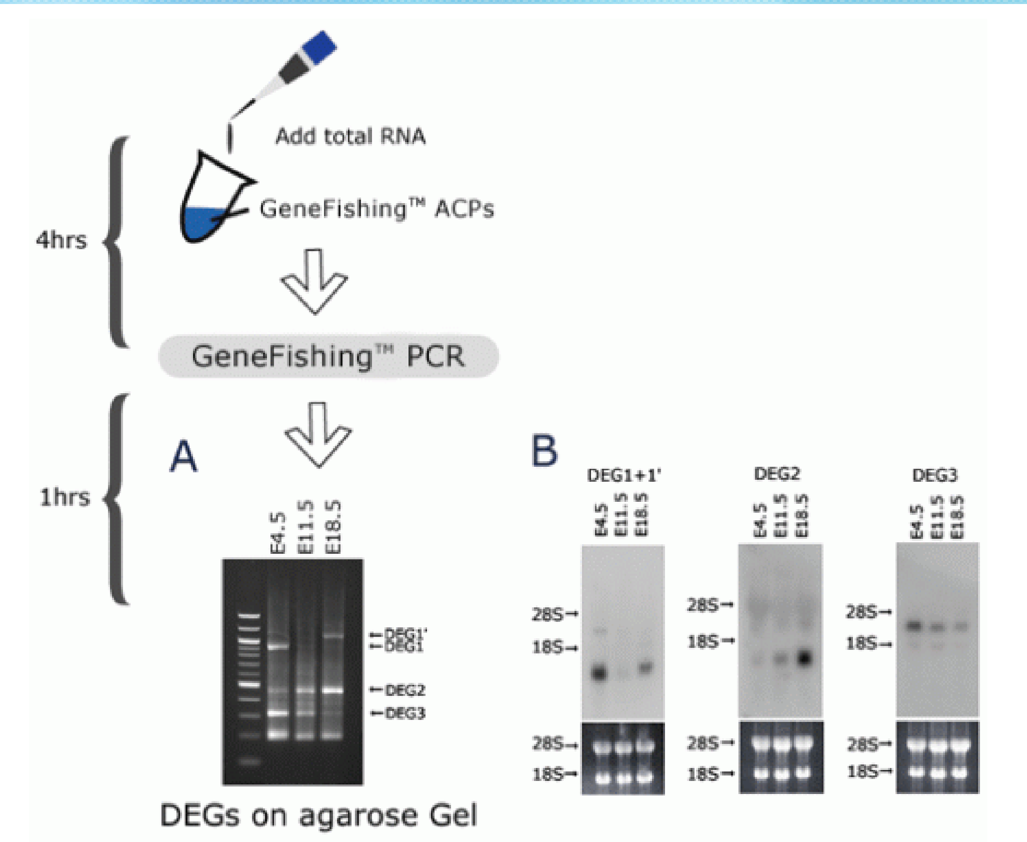
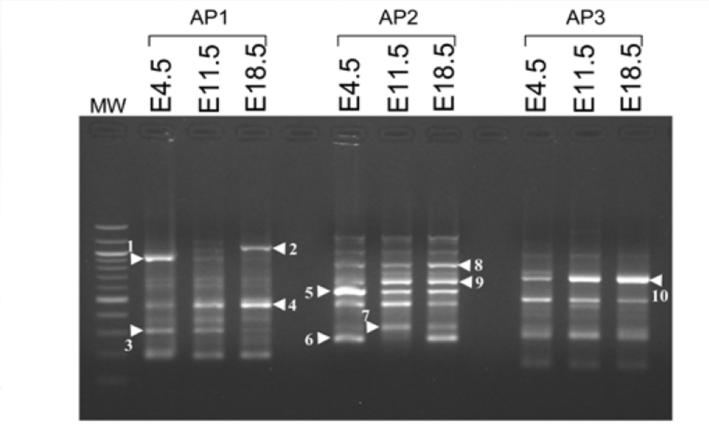


Table 1. Primer Sequences Used in cDNA Synthesis and ACP-Based GeneFishing PCR

Use	Primer	Sequence
cDNA Synthesis Primer	Oligo(dT)$_{15}$	5′-TTTTTTTTTTTTTTT-3′
	Oligo(dT)$_{15}$ tail	5′-CTGTGAATGCTGCGACTACGATTTTTTTTTTTTTTTT-3′
	Oligo(dT)$_{15}$ ACP	5′-CTGTGAATGCTGCGACTACGATIIIIITTTTTTTTTTTTTTT-3′
Arbitrary Primer	10-mer	5′-GCCATCGACC-3′
	10-mer tail	5′-GTCTACCAGGCATTCGCTTCATGCCATCGACC-3′
	AP1	5′-GTCTACCAGGCATTCGCTTCATIIIIIGCCATCGACC-3′
	AP2	5′-GTCTACCAGGCATTCGCTTCATIIIIIAGGAGATGCG-3′
	AP3	5′-GTCTACCAGGCATTCGCTTCATIIIIICTCCGATGCC-3′

ACP, annealing control primer.
The polydeoxyinosine [poly(dI)] linkers are underlined. I represents deoxyinosine.



Objective & Strategy

Objective

❖ 어류의 생산성 관련 유전자 마커 개발
- ➢ 어류의 성장에 관여하는 유전자 탐색
- ➢ 근육 분화 및 발달에 관여하는 유전자 탐색
- ➢ 근육성장 관련 유전자 마커 개발

Strategy

❖ 동일 사육조건에서 성장 차이 나타낸 개체 확보
- ➢ 체장과 체중을 기준으로 성장차이 확인
- ➢ 평균체중이 2배 차이를 나타내는 개체 선별

❖ ACP (Annealing Control Primer)를 이용한 차등발현 유전자 탐색
- ➢ GeneFishing™ DEG kit 사용

Materials & Methods

Experimental fish

- ❖ 강도다리 (Starry flounder, *Platichthys stellatus*)
- ❖ 치어 (평균체중 6.4g)를 19℃에서 56일간 사육
- ❖ 체중이 2배 차이를 보인 개체 선별
  - ➢ Large 그룹
    - ✓ 체중 : 41.0 ~45.0g (평균 43.4g)
    - ✓ 전장 : 14.0 ~15.0cm (평균 14.5cm)
  - ➢ Small 그룹
    - ✓ 체중 : 15.0 ~25.0g (평균 20.0g)
    - ✓ 전장 : 10.3 ~12.4cm (평균 11.5cm)

Materials & Methods

Total RNA 분리 · 정제 및 RT-PCR

❖ 근육, 간, 신장, 아가미, 지느러미, 혈액 total RNA 분리

❖ TRIzol® Reagent 와 chloroform을 사용하여 분리·정제

❖ Total RNA 1㎍을 oligo(dT)$_{18}$ 이용하여 1st cDNA로 전환

ACP (Annealing Control primer) based PCR

❖ GeneFishing ™ DEG Kit (Seegen, Korea) 사용

❖ Large 및 Small 그룹의 근육조직 비교 분석

Materials & Methods

Quantitative real-time RT-PCR analysis

❖ Thermal Cycler Dice™ Real Time System (TaKaRa, Japan)

❖ 각 그룹 근육조직에서 DEG 발현양상 분석

❖ β-actin 유전자 사용하여 normalization

Tissue distribution of starry flounder CKM1

❖ CKM1(creatine kinase muscle type) 유전자 분석

❖ 근육, 간, 신장, 아가미, 지느러미, 혈액에서 발현여부 조사

Materials & Methods

Cloning and sequencing of CKM1 gene

- ❖ SMARTer™ RACE cDNA Amplication Kit
- ❖ 강도다리 CKM1 cDNA 단편의 염기서열 결정

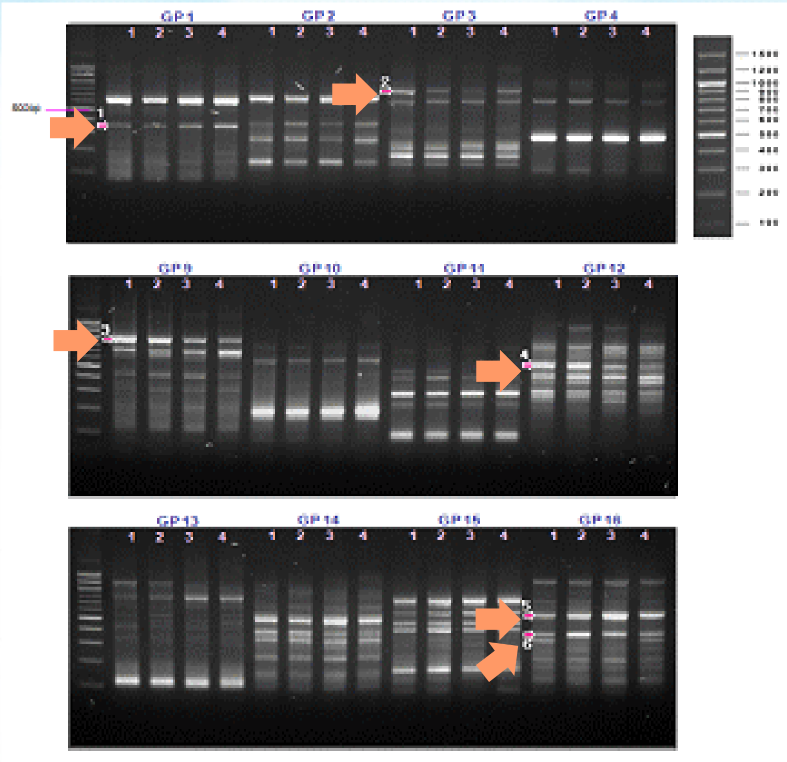
Genomic structure analysis of CKM1 gene

- ❖ 강도다리 CKM1 유전자 genomic clone 분석
- ❖ Exon-intron junction 조사

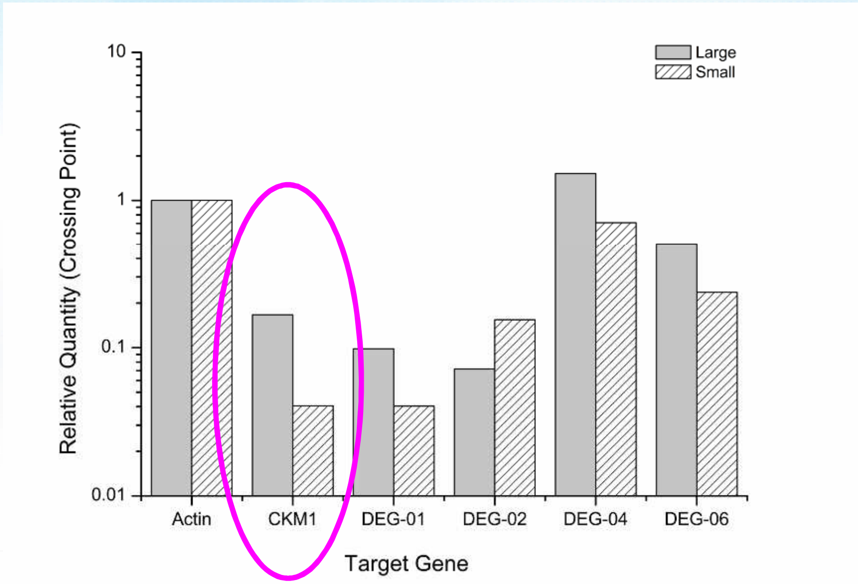
Results

Identification of starry flounder growth-related genes

차등발현유전자 (DEG) 확보



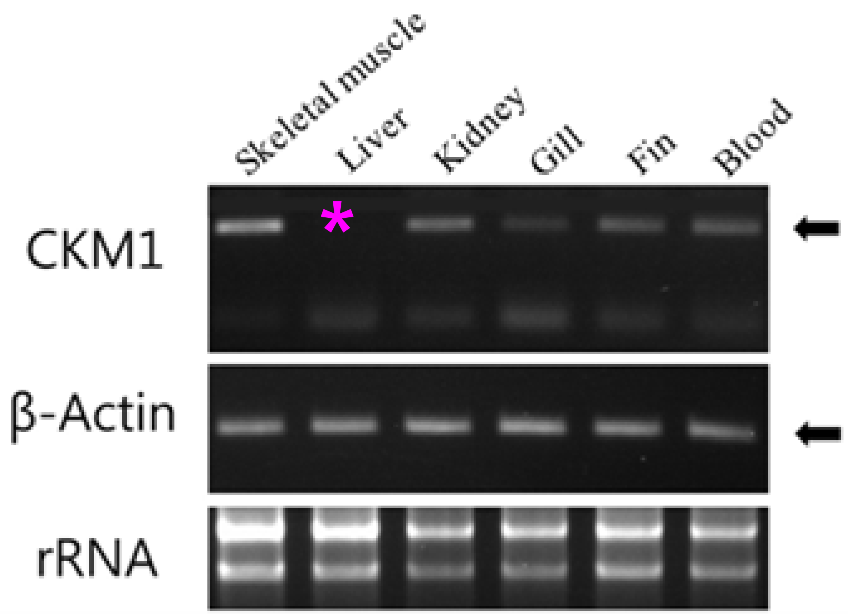
DEG의 발현양상 분석



❖ **CKM1 : large 그룹, 발현량 4.15배**

Results

Tissue expression of starry flounder CKM1 gene



☞ 간조직에서 발현되지 않았음

Results

Characterization of starry flounder CKM1 cDNA

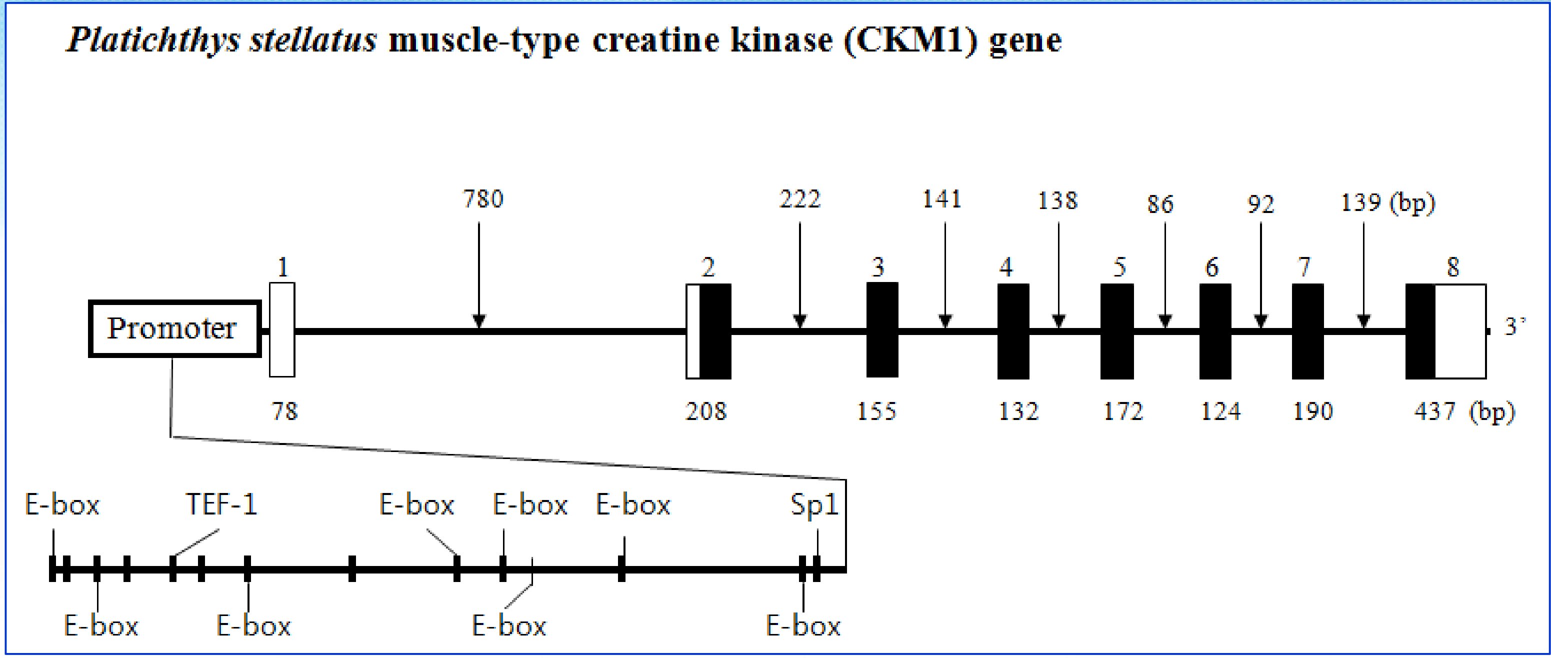
```
   1 GGGGTGCCCCTATTTTGGCTGTGGTGAACAGGATCTGATCCCAAGGACTGTTACCTTTTG
  61 TTCTTGTCTGGTGTGCAGTGTAAGAAAGCAATCATGCCTTTCGGAAACACCCACAACAAC
                                       M   P   F   G   N   T   H   N   N
 121 TTCAAGCTCAACTACAAGGTCGAGGAGGAGTTCCCCGACCTGAGCCTCCACAACAACCAT
      F  K  L  N  Y  K  V  E  E  E  F  P  D  L  S  L  H  N  N  H
 181 ATGGCCAAGGTTCTGACCAAGGAGCTGTATGGCAAGATTAGGGACAGGCAGACACCCAGT
      M  A  K  V  L  T  K  E  L  Y  G  K  I  R  D  R  Q  T  P  S
 241 GGCTACACTGTGGATGATGTCATCCAGACTGGTGTCGACAACCCTGGTCACCCCTTCATC
      G  Y  T  V  D  D  V  I  Q  T  G  V  D  N  P  G  H  P  F  I
 301 ATGACCGTTGGCTGCGTCGCTGGTGATGAGGAGTCCTATGAGGTCTTCAAGGAGCTTCTG
      M  T  V  G  C  V  A  G  D  E  E  S  Y  E  V  F  K  E  L  L
 361 GACCCCATCATCTCAGACCGTCATAATGGATACAAGCCTACTGACAAGCACAAGACCGAC
      D  P  I  I  S  D  R  H  N  G  Y  K  P  T  D  K  H  K  T  D
 421 TTGAACTTCGAGAACCTGAAGGGTGGTGACGACCTGGACCCCAACTATGTTCTGTCCAGC
      L  N  F  E  N  L  K  G  G  D  D  L  D  P  N  Y  V  L  S  S
 481 CGTGTCCGTACTGGACGTAGCATCAAGGGATTCACCCTGCCCCCCCACAACAGCCGTGGC
      R  V  R  T  G  R  S  I  K  G  F  T  L  P  P  H  N  S  R  G
 541 GAGCGCAGAGCTATTGAGAAGCTGTCTGTTGAGGCTCTGGCCAGCCTGGATGGTGAGTTC
      E  R  R  A  I  E  K  L  S  V  E  A  L  A  S  L  D  G  E  F
 601 AAGGGAAAGTACTACCCCCTGAAGTCTATGACTGATGCCGAGCAGGAGCAGCTGATCAGT
      K  G  K  Y  Y  P  L  K  S  M  T  D  A  E  Q  E  Q  L  I  S
 661 GATCACTTCCTGTTCGACAAGCCTGTCTCCCCCCTGCTGACCTGTGCTGGAATGGCCCGT
      D  H  F  L  F  D  K  P  V  S  P  L  L  T  C  A  G  M  A  R
 721 GACTGGCCTGATGCCAGGGGCATCTGGCACAATGAGAACAAGTCCTTCCTGGTCTGGGTC
      D  W  P  D  A  R  G  I  W  H  N  E  N  K  S  F  L  V  W  V
 781 AATGAGGAGGATCACCTGCGTGTCATCTCCATGGAGCAGGGTGGCAACATGAGGGAGGTC
      N  E  E  D  H  L  R  V  I  S  M  E  Q  G  G  N  M  R  E  V
 841 TTCAAGCGTTTCTGCGTTGGCCTTAAAAGGATTGAGGAGATCTTCAAGAAGCACAACCAT
      F  K  R  F  C  V  G  L  K  R  I  E  E  I  F  K  K  H  N  H
 901 GGCTTCATGTGGAACGAGCATCTCGGGTACATCCTGACCTGCCCCTCCAACCTGGGCACT
      G  F  M  W  N  E  H  L  G  Y  I  L  T  C  P  S  N  L  G  T
 961 GGACTGCGTGGTGGTGTCCATGTCAAGCTGCCAAAGCTGAGCACACATCCCAAGTTTGAT
      G  L  R  G  G  V  H  V  K  L  P  K  L  S  T  H  P  K  F  D
1021 GAGATCTCACCAGGCTGCGTCTGCAGAAGCGTGGAACAGGTGGTGTGGACACAGCCTCTG
      E  I  S  P  G  C  V  C  R  S  V  E  Q  V  V  W  T  Q  P  L
1081 TGGGTGGTGTGTTCGACATCTCCAACGCTGACCGTCTGGGCTCCTCTGAGGTGGACCAGG
      W  V  V  C  S  T  S  P  T  L  T  V  W  A  P  L  R  W  T  R
1141 TCCAGCTGGTGGTTGATGGTGTCAAACTGATGGTTGAGATGGAGAAGAAGCTGGAGAAGG
      S  S  W  W  L  M  V  S  N  *
1201 GAGAGGCAGTCGACAGCATGATCCCTGCCCAGAAGTAGAGAGGAACAATCTCATCTTTTT
1261 CCGTGACCATTCATTTATGTTCAACGGAGCCAGCTGATGGCTTTGCAGAGGAAACAGCTG
1321 CTCACCTAGAGACTCTTGACTCCGCTCACCTTTTTTCTCCATACAGCTTTTTCTTTCTTT
1381 CCCCGTCATCATTTTTTTTTTCAAGTTCTCCTGTGTTGGTTGGAAAAATCCCTGGGATCA
1441 CCCCCCACGGGGCTGGGCTCCCCTAGCAAACGGGGCATCCCCCAGTTTTTACAGCTAAAA
1501 ATAATGTTTATTGAAGGGGTTCATATTACTCAAAAAAAAGGGGCCCCGGGAAACAACTAAA
1561 AAAAAAAAAAAAAAAAAAAAAAAAAAAAAA
```

❖ 강도다리 CKM1유전자

- mRNA
  - ✓크기: 1,590 bp
  - ✓ORF: 1개
  - ✓359개의 아미노산

Results

Genomic organization of starry flounder CKM1 gene



❖ 강도다리 CKM1 전체 유전자 크기는 3,325 bp

➢ 8개의 exon과 7개의 intron으로 구성 됨

➢ 조절영역에 E-box 존재

Results

Exon-intron junctions in the starry flounder CKM1 gene

Exon	Size (bp)	Exon-intron juction	Intron	Size (bp)
1	78	.....GGGGTGC.......GTGCAGgtaaag	1	780
2	208	ctgcagTGTAAGA.......ACCCTGgtgaggc	2	222
3	155	atgtttagGTCACCCC.......CTGAAGgtacagt	3	141
4	132	gtttttagGGTGGT.......TTGAGGgtaaac	4	134
5	172	ctgcagCTCTGG........GGCATCTGgtgagta	5	87
6	124	atccagGCACAA.......TAAAAGGgtaaatgt	6	92
7	190	taacagATTGAG.......GAACAGgtatgc	7	138
8	437	ctttagGTGGTG.......AGAAGTAG.....		

☞ Exon-intron boundary는 GT-AG rule을 따르고 있었음

Conclusions

- ❖ ACP-based PCR 방법으로 어류의 성장 관련 유전자 탐색
- ❖ 5개의 차등발현 유전자 확보
  - ➢ 1개는 creatin kinase muscle type (CKM1) gene
  - ➢ 4개는 unknown gene
- ❖ CKM1 유전자 발현양상 분석
  - ➢ 성장 빠른 그룹의 근육조직에서 발현량이 4.15배 많음
  - ➢ 근육,신장,아가미,지느러미 및 혈액에서 발현 확인
  - ➢ 간 조직에서는 발현되지 않았음
- ❖ CKM1 유전자 구조 확인
  - ➢ mRNA는 359개의 아미노산 암호화
  - ➢ 전체 구조는 8개의 exon과 7개의 intron으로 구성
  - ➢ 조절영역에 E-box 존재 확인

감사합니다