Construction of BAC library from XY Japanese flounder using frozen sperm genomic DNA

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What can we do using the genomic information for fish breeding?

- Selection using a commercial trait
- Linkage analysis using genetic markers
- Positional cloning of the responsible gene
- Applying to another fish selection

Whole genome database of model fish
- synteny
- FISH
- physical map
- EST
What fish is our target?

Japanese flounder (*Paralichthys olivaceus*)

- The forth amount of aquaculture production in Japan
- It is delicious, good for *sashimi* but expensive.
- The damage from an infectious disease is huge in aquaculture.
- There are several resistance groups against the disease.
## Genome size of Japanese flounder

<table>
<thead>
<tr>
<th>Species</th>
<th>haploid C-value</th>
<th>Mbp</th>
<th>No. chromosomes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fugu</td>
<td>0.4</td>
<td>400</td>
<td>44</td>
</tr>
<tr>
<td><strong>Japanese flounder</strong></td>
<td><strong>0.71</strong></td>
<td><strong>700</strong></td>
<td><strong>48</strong></td>
</tr>
<tr>
<td>Medaka</td>
<td>0.83</td>
<td>800</td>
<td>48</td>
</tr>
<tr>
<td>Yellowtail</td>
<td>0.83</td>
<td>800</td>
<td>48</td>
</tr>
<tr>
<td>Red seabream</td>
<td>0.93</td>
<td>900</td>
<td>48</td>
</tr>
<tr>
<td>Zebrafish</td>
<td>1.68</td>
<td>1700</td>
<td>48</td>
</tr>
<tr>
<td>Trout</td>
<td>2.07</td>
<td>2000</td>
<td>66</td>
</tr>
<tr>
<td>Human</td>
<td>3.5</td>
<td>3000</td>
<td>46</td>
</tr>
</tbody>
</table>

\[ C = 0.9869 \times 10^9 \text{ bp} \]
Genomic breeding project (FRA) (2003-2005)

- Tokyo Univ. of Mar. Sci. & Tech.
- NRISA
- NRIA
- NRIFS
- Hokkaido Univ.

- Recombination map
  - Microsatellite marker
- DNA resources
  - Genomic library
  - EST analysis
- Physical map
  - Chromosome FISH
1. BAC library from frozen sperm of XY heterozygous flounder

2. Screening using the BAC library

3. Feature of MHC class Ia cluster
#1. BAC library from frozen sperm of XY heterozygous flounder
How to check heterozygousity of paternal fish
Estradiol treatment and sex ratio of offspring from male fish TY-4

Mild 10 μg/l of estradiol treatment

↓

Be male

XY genetic male

Phenotypic male

♂ 61 53%

Repress sex reversal

XX genetic female

Phenotypic female

♀ 54 47%
Freezing and defrosting of sperm

We corrected semen from five male fish, and centrifuged that to obtain a sperm pellet in June 2003.

We frosted the sperm pellet in liquid nitrogen and kept it in a freezer at –80°C until October 2003 or December 2003 (for 4-6 months).
Insert size of BAC clones of heterozygous Japanese flounder

Ave. 140.7 kb
Size of TY-4 XY BAC library

• 110,592 BAC clones were picked and arrayed in 288 of 384-well microtiter plates.

• An average insert size of 140.7 kb was obtained by the present analysis.

• The calculations predicted a 22.2-fold (700 Mbps) coverage of the Japanese flounder genome.
Summary #1

1. **Frozen sperm** is useful in constructing BAC and library. It facilitates the preparation of a high-molecular DNA sample, and the construction of genomic libraries.

2. We have produced a **heterozygous (XY)** genome resource of Japanese flounder as a BAC library.
#2. Screening using the BAC library
Objectives

- Evaluate the screening efficiency of the BAC library

What are our targets?

- Major histocompatibility complex (MHC) cluster, which is supposed to be related with the resistance of disease.

- The 24 recombination linkage markers, which will correspond to each chromosomes.
MHC （Major histocompatibility complex）

- MHC class I （I a, I b）
  α chain & β2-microglobulin
  Polymorphic domain: α1 & α2
  Present antigen to cytotoxic T-cell (CD8+)

- MHC class II （II α, II β）
  α chain & β chain
  Polymorphic domain: α1 & β1
  Present antigen to helper T-cell (CD4+)
MHC(HLA) gene cluster

- There are many genes supposed to be related with an immune system.

- The gene density in the cluster is very high (16-18 kbp /gene).

- About 20 genes each 400kbp are found in this gene cluster in model fishes.
MHC genes reported from EST analysis of Japanese flounder

Class Ia: Paol-UA1, -UA2, -UA3, -UA4, -UA5
   In all tissues. Present antigen to cytotoxic T-cell

Class Ib: Paol-UB1
   In lymphoid organs, gill, intestine or liver.
   Present antigen to NK cell (?)

Class IIα: Paol-D(01)A, -D(02)A
   In all tissues. Present antigen to helper T-cell

Class IIβ: Paol-D(01)B, -D(02)B
   In all tissues. Present antigen to helper T-cell
Results of screening for MHC genes

<table>
<thead>
<tr>
<th>MHC</th>
<th>Clone number</th>
<th>Clone number</th>
<th>Clone number</th>
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</thead>
<tbody>
<tr>
<td>Class Ia</td>
<td>20G10</td>
<td>52L18</td>
<td>55I6</td>
</tr>
<tr>
<td>Class Ib</td>
<td>59C10</td>
<td>83O20</td>
<td></td>
</tr>
<tr>
<td>Class II α</td>
<td>15P9</td>
<td>56M13</td>
<td></td>
</tr>
<tr>
<td>Class II β</td>
<td>15P9</td>
<td>56M13</td>
<td></td>
</tr>
</tbody>
</table>

From 1/3 plates of library (5.9-folds coverage)
Three positive clones including MHC class Ia

<table>
<thead>
<tr>
<th>Marker</th>
<th>23</th>
<th>9.4</th>
<th>6.5</th>
<th>4.3</th>
<th>2.3</th>
<th>2.0</th>
<th>kb</th>
</tr>
</thead>
<tbody>
<tr>
<td>55I6</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>52L18</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>20G10</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>20E10</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<td></td>
</tr>
</tbody>
</table>

EcoRI digest pattern

52L18 ≠ 55I6 > 20G10
Check sequence of MHC class Ia

The PCR fragments amplified from the positive clones

- **52L18-204bp**
  
  GCGAGCTGAAACACACAGTCGTACCTCCTCCTCCAGTCTTTTCAAGGTTGGGATTGATGAAACCTGCAGGTCAGCTGCTCATCTGGAAGGTCCCGTCATGTTTG
  
  GGGAGGACCTCTCCGACGTACACGTCCTCCTCATGAAGCTCCTCTCCGTC
  
  TTCTCCAGAACAACATGGGTAGTAGACCGTAGACCTGACTGGA

- **55I6-204bp**
  
  GCGAGCTGAAACACACAGTCGTACCTCCTCCTCCAGTCTTTTCAAGGTTGGGATTGATGAAACCTGCAGGTCAGCTGCTCATCTGGAAGGTCCCGTCATGTTTG
  
  GGGAGGACCTCTCCGACGTACACGTCCTCCTCATGAAGCTCCTCTCCGTC
  
  TTCTCCAGAACAACATGGGTAGTAGACCGTAGACCTGACTGGA
Correspond linkage groups to chromosomes

**Linkage map**
- 24 Linkage groups

**Screening**

**Physical map**
- 24 Chromosomes

24 BAC clones

BAC library

Corresponding

FISH probe
Summary #2

1. We screened two or three positive clones for all four-subclass of MHC genes from the BAC library of Japanese flounder.

2. The PCR fragments of 52L18 and 55I6 clones indicate the sequences of MHC class Ia gene.

3. We have screened 24 markers of each linkage groups from the library of frozen sperm, it shows that freezing does not create any bias in the library.
#3. Feature of MHC class Ia cluster
Objectives

- Analyze the synteny in MHC cluster between the model fish and Japanese flounder

- Develop the linkage marker of MHC cluster, and map in the linkage group.
Presumptive genes in eight contigs of 52L18

- MHC class Ia—UA4 and UA5
- TCF19
- Contactin-binding protein
- Dynactin
- Hepatitis C virus genome polyprotein
- Regulating synaptic membrane exocytosis 1
- VHSV-induced protein (rainbow trout)
- Metallothionein-like
ORF prediction by GENESCAN and BLAST search
Position of MS(CA)-repeat over eight times

<table>
<thead>
<tr>
<th></th>
<th>Number of MS</th>
<th>Number of genes</th>
<th>Density of genes</th>
</tr>
</thead>
<tbody>
<tr>
<td>flounder</td>
<td>30/95 kb (3.2 kb/MS)</td>
<td>8/95 kb</td>
<td>11.9 kb/gene</td>
</tr>
<tr>
<td>medaka</td>
<td>69/425 kb (6.2 kb/MS)</td>
<td>23/425 kb</td>
<td>18.5 kb/gene</td>
</tr>
</tbody>
</table>

Gene density in MHC of human 16kb (224 loci/3.6 Mb)
Summary #3

1. There is a **four-tandem-repeat** of MHC class Ia gene in a BAC clone.

2. The class Ia region of the flounder indicates the **high density** of microsatellite as much as that of medaka.
Total Summary

- **Frozen sperm** is useful for preparation of a high-molecular DNA sample, and construction of genomic (BAC, cosmid) library.

- Screening data indicates that freezing does **not** create any bias in BAC library.
In Future

Integrate genomic information using a radiation hybrid (RH) panel(map).
Genomic breeding project (FRA), 2003-2005

Recombination map
- Microsatellite marker

DNA resources
- Genomic libraries
- EST analysis

Physical map
- Chromosome FISH

Radiation hybrid (RH) panel, 2005-

RH map (Integration of MS, EST, SNP, BAC and etc.)
→ access to genomic structural information of other organisms
→ get a clue of positional cloning or marker development
→ will be a powerful tool for proceeding of genomic breeding