(Form4-2)

JOINT RESEARCH PROJECT

FINAL REPORT
For Japan-Korea Joint Research Project

AREA	1. Mathematics & Physics 2. Chemistry & Material Science ③. Biology 4. Informatics & Mechatronics 5. Geo-Science & Space Science 6. Medical Science 7. Humanities & Social Sciences

1. Research Title:

Diagnosis of physiological status of marine rotifers utilizing molecular biomarkers

2. Term of Research: From July 2009 To June 2011

3. Total Budget

a. Financial Support by JSPS: Total amount: 2,400 thousand yen

1st Year 600 thousand yen 2nd Year 1,200 thousand yen

3rd Year 600 thousand yen

b. Other Financial Support : Total amount: 200 thousand yen

4. Project Organization

a. Japanese Principal Researcher	
Name	Atsushi HAGIWARA
Institution / Department	Graduate School of Fisheries Science and Environmental Studies, Nagasaki University
Position	Proferssor
b. Korean Principal Researcher	
Name	Jae-Seong LEE
Institution / Department	Department of Chemistry, Hanyang University
Position	Professor

c. List of Japanese-side Participants (Except for Principal Researcher)

Name	Institution/Department	Position
Kiyoshi SOYANO	Institute for East China Sea Research, Nagasaki University	Professor
Yoshitaka SAKAKURA	Graduate School of Fisheries Science and Environmental Studies, Nagasaki University	Professor
Koushirou SUGA	Graduate School of Fisheries Science and Environmental Studies, Nagasaki University	Associate Professor
Hee-Jin KIM	Graduate School of Science and Technology, Nagasaki University	PhD student

d. List of Korean-side Participants (Except for Principal Researcher)

Name	Institution/Department	Position
Heum Gi PARK	Faculty of Marine Bioscience and Technology, Kangnung National University	Professor
Hans-Uwe DAHMS	Dept of Chemistry, Hanyang University	Research Professor
Kyun-Woo LEE	Dept of Chemistry, Hanyang University	Research Assistant Professor
Dae-Sik Hwang	Dept of Chemistry, Hanyang University	PhD student
Jae-Sung Rhee	Dept of Chemistry, Hanyang University	PhD student

5. Number of Exchanges during the Final Fiscal Year*

a. from Japan to Korea

*Japanese fiscal year begins April 1.

Name	Home Institution	Duration	Host Institution
Atsushi HAGIWARA	Nagasaki University	6.16.2011-6.18.2011	Hanyang University
Yoshitaka SAKAKURA	Nagasaki University	6.16.2011-6.18.2011	Hanyang University
Koushiro SUGA	Nagasaki University	6.16.2011-6.18.2011	Hanyang University

For Final Fiscal Year(FY2011)	For Final Fiscal Year(FY2011)
Total: 3 persons	Total: 9 man-days

Numbers of Exchanges during the past fiscal years
FY2009: Total 4 persons FY2010: Total 3 persons

b. from Korea to Japan

Name	Home Institution	Duration	Host Institution

For Final Fiscal Year(FY2011)	For Final Fiscal Year(FY2011)
Total: 0 persons	Total: 0 man-days

Numbers of Exchanges during the past fiscal years
FY2009: Total 3 persons FY2010: Total 1 persons

6. Objective of Research

This project aims at developing molecular biomarkers for detecting stress of marine zooplankton population, so that rapid diagnosis of physiological status of the zooplankton feasible.

We employ monogonont rotifer *Brachionus plicatilis* (phylum Rotifera) as test species, because it is important zooplankton species in the food web of coastal brackish waters. Due to its comparative easiness of culturing, this species has been utilized world-wide as a live food for feeding early life stage of marine fishes in aquaculture industry. The lack of methods to detect and recover physiological conditions of mass-cultured rotifers have been bottleneck of stable production of marine fishes in world-wide hatcheries. This rotifer species has also been recognized as a ideal test organism for assessing toxicity of contaminants in natural waters due to its short life history.

The Japanese team has conducted relevant research activities with regard to reproduction, population dynamics and behavioral ecology of rotifers, but their molecular mechanisms have not been clarified, which limits the availability of this species in aquaculture and environmental industry. Japanese team recently succeeded in constructing a database of 7000 ESTs as well as sequencing and analyzing entire structure of mitochondrial genome of this species for the first time in the world, which can be a basis to understand the rotifer life cycle at molecular level.

In the mean time, Korean team developed advanced technique in sequencing ESTs to develop oligoChip that can be applied to detecting stress of marine fish and crustacean. By jointing research activity of both teams, we will be able to obtain comprehensive basic knowledge about molecular status of rotifers in stressed environment first time in the world.

7. Methodology

***Brachionus plicatilis* male cDNA library**

A normalized cDNA library was constructed using about 7,000 males from axenic culture of *B. plicatilis* NH1L strain, which were harvested by filtration, and the contaminating small females and neonates were removed by hand pipetting. The cDNA library was constructed using the SMART cDNA library construction kit. The Duplex Specific Nuclease was used to eliminate highly expressed genes.

Isolation of dormancy breaking genes from resting eggs

The total RNAs, which were isolated from resting eggs after 0- 30-min and 4-h light illumination, were used to construct the cDNAs. The reverse transcription differential display-PCR analysis was performed with these cDNAs using the Delta Differential Display Kit. The differentially expressed gene fragments were cloned and sequenced. All sequence data were compared to NCBI databases using BLASTIN and BLASTX. Putative function was assigned based on best BLAST hit to a well-annotated sequence.

Microarray development and analysis

The oligomicroarray development and data analysis was performed at the E-Biogen Inc. (www.e-biogen.com, Seoul, South Korea). The Agilent eArray platform was used to design a rotifer microarray, and we successfully designed 60-mer probes using eArray software (http://earray.chem.agilent.com/earray). For the microarray experiment, total RNAs were isolated from rotifer using conventional TRIzol™ reagent (Invitrogen, USA), and the integrity of isolated total RNA was measured with Agilent's Bioanalyzer 2100 RNA Nano kit (Agilent Technologies). For control and test RNAs, the synthesis of target cRNA probes and hybridization were performed using Agilent's Low RNA Input Linear Amplification kit PLUS according to the manufacturer's instructions. Briefly, each 1 μg of total sample RNA and T7 promoter primer mix was mixed and incubated at 65°C for 10 min. cDNA master mix (5 × first strand buffer, 0.1 M DTT, 10 mM dNTP mix, RNase-Out, and AffinityScript-RT) was prepared and added to RNA and primer mixture. The samples were incubated at 40°C for 2 h for reverse-transcription and double-strand cDNA (dsDNA) synthesis and terminated by incubating at 65°C for 15 min. The transcription master mix was prepared as the manufacturer's protocol (4xTranscription buffer, 0.1 M DTT, NTP mix, 50% PEG, RNase-Out, Inorganic pyrophosphatase, T7-RNA polymerase, and Cyanine 3-CTP) and added to the dsDNA reaction mixture and incubated at 40°C for 2 h for transcription of dsDNA. During transcription-amplification, control and test cRNAs were labeled with Cy3-CTP. Amplified and labeled cRNA was purified and quantified using ND-1000 spectrophotometer (NanoDrop Technologies, Inc., Wilmington, DE) to check labeling efficiency, followed by fragmentation of cRNA performed by adding 10xblocking agent and 25xfragmentation buffer and incubating at 60°C for 30 min. The fragmented cRNA was resuspended with 2xhybridization buffer and directly pipetted onto microarray placed in a hybridization chamber (Agilent Technologies, USA). By incubating the hybridization chamber at 42°C for 16 h at mild agitation, the hybridization reactions took place between labeled targets and probes on the microarray. To eliminate non-specific binding, the hybridized microarrays were washed with Agilent's Gene Expression Wash Buffer Kit (Agilent Technologies). Finally, microarrays were spin-dried and stored in dark condition until scanning. Scanned images were analyzed by Feature Extraction program (Agilent Technologies). The average fluorescence intensity for each spot was calculated and the local background was subtracted. All data manipulation and selection of fold-changed genes was performed using GeneSpring 7.3.1 (Agilent Technologies). Array points with less than 0.01 signal intensity were transformed to 0.01 (data transformation), and the intensity of the test channel was subtracted by the control channel to calculate the normalized ratio values for each spot. Genes that changed for more than 2-fold were selected and considered as genes with significant information. The gene ontology (GO) functional annotations (Suppl. S2-S4) and the KEGG pathway mapping were performed at the NICEM, Seoul National University (Seoul, South Korea).