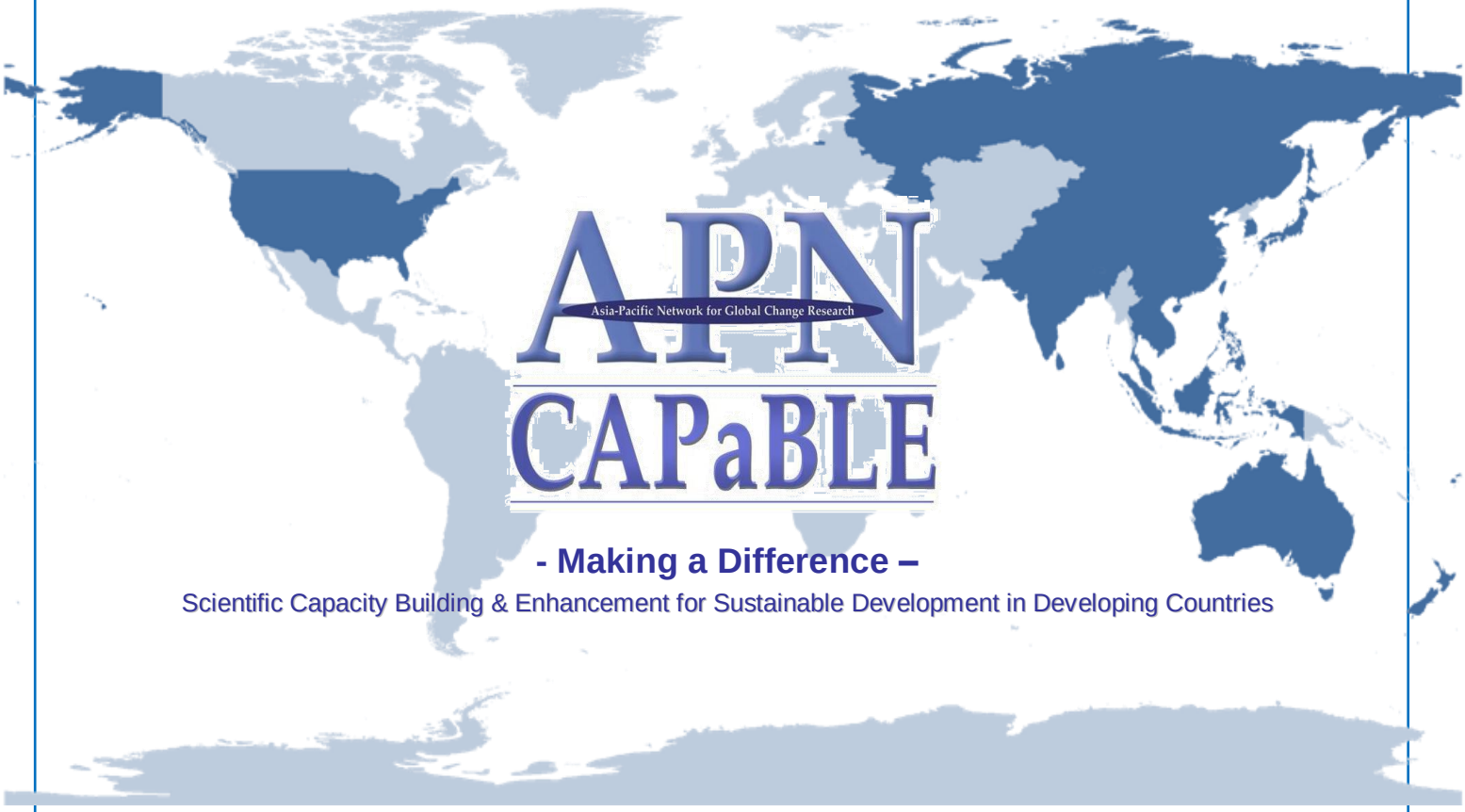
FINAL REPORT for APN PROJECT

Project Reference Number: CBA2011-01CMY-Kawai

Capacity Building of Biodiversity Research in the Coastal Zones of the Asia Pacific Region:

Phycology Taxonomy Analysis Training Using Genetic Marker



The following collaborators worked on this project:

Project Leader; Hiroshi Kawai, Kobe University, Japan, Kawai@kobe-u.ac.jp

Collaborator; Takeo Horiguchi, Hokkaido University, Japan, horig@sci.hokudai.ac.jp

Collaborator; Kazuhiko Kogame, Hokkaido University, Japan, kogame@sci.hokudai.ac.jp

Collaborator; Mitsunobu Kamiya, Fukui Prefectural University, Japan, mkamiya@fpu.ac.jp

KOBE UNIVERSITY

HOKKAIDO UNIVERSITY FOUNDED 1876

Capacity Building of Biodiversity Research in the Coastal Zones of the Asia Pacific Region: Phycology Taxonomy Analysis Training Using Genetic Marker

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OVERVIEW OF PROJECT WORK AND OUTCOMES

Non-technical summary

This project aimed to provide practical trainings of investigation techniques for the molecular phylogenetic analyses to the researchers of Southeastern Asia. Furthermore, we aimed to establish regional network of researchers for developing research collaborations in the study of non-indigenous marine algae, because exchange of taxonomic and genetic information of local populations in the adjacent areas is especially important in studying non-indigenous taxa.

By the financial aid of APN center and International EMECS center, and also by kind support of Asian Pacific Phycological Association (APPA) and some local members of the North Pacific Marine Science Organization (PICES), we could invite six young enthusiastic trainees every two years from Cambodia, China, Indonesia, Malaysia, Thailand and Vietnam. They were researchers or graduate students who are already working on some algal taxa, so that, in order to make the program more practical for each trainee, we suggested carrying and using their own research materials in the practice. And the instructors were all algal taxonomists actively at work leading phycological research in Japan. Under such an ideal combinations, in spite of the short period of the program, we believe the trainees could learn essential part of the techniques, and could have fruitful scientific discussions with the instructors for adopting the new methods for solving the problems in their own research topics.

Objectives

The main objectives of the project were:

1. To inform the aim and purpose of the program through orientation and explanation as to the future influence and development of this program.
2. To learn phycology taxonomy analysis techniques using genetic marker method from the basic level to a practical level through laboratory practice.
3. To exchange their training results and comments each other.
4. To steadily development their network in the future.

Amount received and number years supported

The Grant awarded to this project was:
US$ 30,000 for Year 1 (2010)
US$ 30,000 for Year 2 (2011)
US$ 60,000 in total

Activity undertaken

The number of trainees: 6 participants in each year.

2010

Han XIAOTIAN (China) (Institute of Oceanology, Chinese Academy of Science).
Roike Iwan MONTOLALU (Indonesia) (Fisheries and Marine Science, Sam Ratulangi University).
Woan-Shien NG (Malaysia) (Institute of Biological Sciences, Institute of Ocean and Earth Sciences, University of Malaya).
Anchana PRATHEP (Thailand) (Department of Biology, Faculty of Science, Prince of Songkla Unviersity).
Soradkorn PHIMLA (Thailand) (Algal Bioresorces Research Center, Department of Fishery Biology, Faculty of Fisheries, Kasetsart University).
Minhthanh Thi NGUYEN (Vietnam) (Algal Biotechnology Department, Institute of Biotechnology, Vietnamese Academy of Science and Technology).

2011

Phala CHEA (Cambodia) (Marine Aquaculture Research and Development Center).

Tsz Yan NG (China) (Marine Science laboratory, School of Life Sciences, The Chinese University of Hong Kong).
Phaik Eem LIM (Malaysia) (Algae Research Laboratory, Institute of Ocean and Earth Science, University of Malaya).
Sze-wan POONG (Malaysia) (Algae Research Laboratory, Institute of Ocean and Earth Science, University of Malaya).
Narongrit MUANGMAI (Thailand) (Department of Fishery Biology, Faculty of Fisheries, Kasetsart University).
Ngocmai Thi DINH (Vietnam) (Algal Biotechnology Department, Institute of Biotechnology, Vietnam Academy and Science and Technology).
(see the Appendix 2)

The number of instructor: 6 instructors who are all algal taxonomists in Japan.
Hiroshi KAWAI (Kobe University Research Center for Inland Seas, 1-1 Rokkodai-cho, Nada-ku, Kobe, Japan).
Takeaki HANYUDA (Kobe University Research Center for Inland Seas, 1-1 Rokkodai-cho, Nada-ku, Kobe, Japan).
Akira KURIHARA (Kobe University Research Center for Inland Seas, 1-1 Rokkodai-cho, Nada-ku, Kobe, Japan).
Takeo HORIGUCHI (Department of Natural History Sciences, Faculty of Science, Hokkaido University, North 10, West 8, Sapporo, Japan).
Kazuhiro KOGAME (Department of Natural History Sciences, Faculty of Science, Hokkaido University, North 10, West 8, Sapporo, Japan).
Mitsunobu KAMIYA (Faculty of Marine Biosciences, Fukui Prefectural University, 1-1 Gakuen-cho, Obama, Fukui, Japan).

The invited commentator (2010).
Prof. Anong CHIRAPART (Department of Fishery Biology, Faculty of Fisheries, Kasetsart University, Thailand).

Host laboratories:
Kobe University Research Center for Inland Seas.
Anchana PRATHEP and Woan-Shien NG (2010).
Phaik Eem LIM and Narongrit MUANGMAI (2011).
Department of Natural History Sciences, Faculty of Science, Hokkaido University.
Xiatian HAN and Minhthanh Thi NGUYEN (2010).
Tsz Yan NG and Sze-Wan POONG (2011).
Faculty of Marine Bioscience, Fukui Prefectural University.
Soradkorn PHIMLA and Roike Iwan MONTOLALU (2010).
Ngocmai Thi DINH and Phala CHEA (2011).

Organizers: APN Center and International EMECS Center.

Selection of trainees:
Applicants: 10 people in 2010
8 people recommended from APPA (Asian Pacific Phycology Association)
3 from Thailand, 2 from Malaysia, 1 from China, 1 from Indonesia and 1 from Vietnam
2 people recommended from PICES (the North Pacific Marine Science Organization)
2 from China
and 11 people in 2011

10 people recommended from APPA (Asian Pacific Phycology Association)
2 from Cambodia, 2 from China, 2 from Malaysia, 2 from Vietnam, 1 from Philippine, and 1 from Thailand
1 person recommended from PICES (the North Pacific Marine Science Organization)
1 from Philippine

The member of selection of trainees: 3 specialists on algal taxonomy.
Prof. Dr. Hiroshi Kawai (Kobe University Research Center for Inland Seas, Japan).
Prof. Dr. Phang Siew Moi (Institute of Biological Sciences, University of Malaya, Malaysia).
Dr. Li Zheng (Marine Ecology Center, First Institute of Oceanography, SOA, China).

6 trainees in each year as mentioned before pages were selected from all applicants by the member of selection of trainees.

Main activities of the project were as below:
Training program consists of 3 parts (see the Appendix 1)
Comprehensive lecture (one day)
Trainees are informed of the aim and purpose of the program through orientation and explanation as to the future influence and development of this program.
Individual training practice (six days)
Six days of training for mastering basic skills in identification using genetic marker from its basic to application, with one-on-one hands-on training in three institutions: Kobe University, Hokkaido University and Fukui Prefecture University.
Summary workshop (one day)
To hold a summary workshop bringing together all trainees to exchange results and comments.

Trainees Nominee Qualifications (extract from the guideline for applicants)
Current Duties; officer, technical officer, engineer, researcher, educator in the administrative bodies
Experience; more than 3 years' experience
Educational background; equal or surpassing to a doctor's degree
Age; between the ages of 26 and 40 years
Health; must be in good health, both physically and mentally

Results

Trainees were informed of the aim and purpose of the program through orientation and explanation as to the future influence and development of this program on the comprehensive lectures from organizer, International EMECS Center, and 5 instructors, Prof. Kawai, Prof. Horiguchi, Prof, Kamiya, Dr. Hanyuda and Dr. Kurihara.

For mastering basic skills in identification using genetic marker from its basic to application, they learned the processes of phycology taxonomy analysis techniques by individual training practice for six days. Training program included: (1) DNA extraction, (2) purification of DNA, (3) agarose gel electrophoresis, (4) PEG precipitation, cycle sequencing, (5) purification of cycle sequencing products, (6) electrophoresis, (7) aligning sequences, (8) phylogenetic analysis, (9) PCR technique, and (10) culturing technique.

On the summary meeting they exchanged their practical results and comments to practices with instructors and each other.

The training schedule was quite limited, but all trainees certainly got the new knowledge and skills from this training course. Furthermore, this training provided the opportunity for dialogue with other participants from different communities and cultures. The network in this field in Southeast Asia and Japan will be built in the near future.

Relevance to the APN Goals, Science Agenda and to Policy Processes

In order to achieve sustainable development conserving the marine biodiversity, effective prevention of the spread of alien species caused by economical activities such as fishery and ship transport is essential. Once the introduced non-indigenous species become settled and spread in some area (country) it is difficult to eliminate them, so that early detection of them is important. However, because of scanty taxonomic information of marine organisms in developing countries, and shortage of taxonomy specialists, it is rather difficult to identify marine organisms in conventional taxonomy methods. Especially for seaweeds, because of their limited morphological features, and remarkable morphological plasticity, it is difficult to identify the species.

This project plays a significant role in the field of monitoring and rapid assessment. Through capacity building of young researchers among APPA (Asian Pacific Phycological Association) countries covering large of the APN countries in the field of taxonomy identification using genetic marker is estimated to cultivate the monitoring network background for the rapid assessment of alien species from other regions.

Self evaluation

Because of the limited number of phycologists (algal researchers including seaweed taxonomists) in some of the target countries of the project (i.e., Philippines, Malaysia, Indonesia, Cambodia), there are no established phycological community or society. Therefore, there was some difficulty in the processes of advertising the project and selecting participants from the applicants. However, by the kind support of APPA secretariat, we believe our selections were appropriate.

The number of trainees was not large, and we regret that we could not accept many of the applicants, but considering the efficiency of the practice and the load to the laboratories that accepted the trainees, it was reasonable number. The training period was somewhat short (and this was commented by some trainees after the course), but I think this was also difficult to expand considering the load to the instructors and laboratories. Therefore, in general, I think the project was successful, and it will be important to make efforts to maintain the relationship with the host laboratories (instructors) and the trainees to support the trainees to establish and develop molecular works in the home institutes.

Potential for further work

The problem of non-indigenous marine species is becoming more emergent and serious by the development of mariculture and ship transport in the area. The molecular technic for identifying marine species is applicable for diverse research topics, and once the technic becomes a routine research technic in the laboratory of the trainees, it is not difficult to teach from one member of the laboratory to new members. On the other hand the molecular biological techniques are developing very rapidly and diverse new research instruments are invented and commercialized. Therefore, it will be helpful for the trainees to provide some opportunity to update their knowledge and technique after some years.

The project was helpful to make networks between the applicants. Therefore, some programs to support the development and continuations of the regional networks (e.g. symposium on the related topics in one of the countries of the trainees) will be effective.

Acknowledgments

At the end of this training program, we would like to acknowledge our sincere thanks to Prof. Siew Moi Phang (University of Malaya, Malaysia) and Prof. Li Zheng (First Institute of Oceanography, Quindao) for their help in the selection process of the trainees, and to Prof. Anong Chirapart (Kasetsart University, Thailand) for visiting Japan of the program, and her lecture and helpful comments to the reports of the trainees.

TECHNICAL REPORT

Preface

Taxonomy is regarded as one of the bases of Biodiversity, and is required to establish certain objective standards to identify any alien species with conventional morphological approaches. In this sense, identification methodology using genetic markers is recognized to reinforce the shortcomings of traditional approaches.

This project aims to contribute to the United Nations Convention of Biodiversity (UNCBD) through,

- Facilitating taxonomical information exchange among young researchers from South East Asian countries.
- Promoting networks with groups engaged in traditional morphological taxonomy.
- Seeking involvement with the network of PICES, for example, to foster information exchange.

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1.0 Introduction

Introductions of non-indigenous seaweeds (macroalgal species) are considerable threat to local coastal ecosystem and local fishery. However, taxonomic information of seaweeds in Southeastern Asia is rather limited, because of the shortage of experienced taxonimists in the region. Furthermore, even for specialists, precise species level taxonomy of seaweed species is often difficult due to their morphological plasticity. On the other hand, molecular phylogenetic approaches are useful research tools for the taxonomy of seaweeds, even for researchers who have limited morphological knowledge of the taxa. Furthermore, genetic markers can be used for detecting the introductions of non-indigenous populations of some native species, including target species of mariculture.

We invited six young enthusiastic trainees every two year from Cambodia, China, Indonesia, Malaysia, Thailand and Vietnam. They were researchers or graduate students who are already working on some algal taxa, so that, in order to make the program more practical for each trainee, we suggested carrying and using their own research materials in the practice. And the instructors were all algal taxonomists actively at work leading phycological research in Japan. Under such an ideal combinations, in spite of the short period of the program, we believe the trainees could learn essential part of the techniques, and could have fruitful scientific discussions with the instructors for adopting the new methods for solving the problems in their own research topics.

Introductions of alien macro algal species associated with globalization of marine transportations, fishery activities, and global climate change, have become more frequent and are a considerable threat to local coastal ecosystems. However, the traditional taxonomy of marine macroalgae, morphological analysis, is not expected to work properly, because there are very few trained macro algal taxonomists in most countries of the Asia-Pacific region. (Requires good knowledge of algal taxonomy with long period of experience for precise identification.)

On the other hand, it has been recognized that using DNA sequence data of slowly evolving gene regions can help identify the preliminary classification of samples in higher taxonomic ranks (e.g. *rbc*L sequences for order and family level), and that of higher evolving genes (e.g. mitochondrial genes and their non-coding regions) and provide information for generic and species level identification.

This project is, through the above DNA analysis, targeted to cover the knowledge and experience of macro algal taxonomy to young researchers for the precise identification skill to distinguish native and alien seaweed species in the Asia-Pacific region.

In general, although the period of preparation as well as the actual practice courses was rather short, the program was productive, and the communications among all participants also developed. The good friendship and academic relationship established during the program and help to strengthen the network.

2.0 Methodology

Training program consists of 3 parts, the comprehensive lecture in the introduction meeting, individual training for mastering basic skills in three institutions and summary workshop.

2.1 Comprehensive lecture

Trainees are informed of the aim and purpose of the program through orientation and explanation as to the future influence and development of this program.

Take lectures on taxonomic study of macroalgae using molecular markers and morphology from four instructors.

2.1.1 Taxonomy of marine macroalgae using molecular markers, by Prof. Mitsunobu Kamiya, Fukui Prefectural University

Generally macroalgal species have much less diagnostic characters to distinguish than animals and land plants, and furthermore show high morphological plasticity induced by biotic/abiotic factors., making it difficult to classify and identify them precisely. Under these background, molecular phylogenetic analysis that have been developed during this two decades have greatly progresses taxonomic and phylogenetic studies on macroalgae since using molecular markers are advantageous to systematic studies. Firstly, there are many "housekeeping" genes that are indispensable for most organisms, and their evolutionary relationships can be analyzed using such gene sequences even if there are few morphological information to compare. Secondly, inter- and intraspecific variation can be comprehensively detected using genes with high evolutionary rate. Thirdly, DNA sequences are not affected by environmental variations and developmental stages that can cause morphological change. Fourthly, because there are only four variations for nucleotides (20 variations for amino acids), it is easy to define theoretical models and to achieve statistical analysis based on molecular data sets. Finally, if DNA substitutions are assumed to occur at a fixed rate, one can use such a "molecular clock" to estimate divergence time.

Today I will introduce a taxonomic study on green macroalgae *Ulva* species from various salinity regions in Mikata-goko, an area consisting of five lakes that are directly or indirectly connected to the sea, resulting in salinity variation throughout. Although it is well known that some members of *Ulva* appear in brackish regions, their taxonomy has not been well understood due to their high morphological plasticity in such brackish waters. When we collected bimonthly ten specimens from each of five sites showing different salinity regimes and sequenced plastid *rbc*L gene, total six haplotypes were distinguished: four of them were phylogenetically close to *U. prolifera*, *U. flexuosa* or *U. limnetica* but the other two haplotypes were possibly new species. Although these six haplotypes were not always distinguishable by conventional morphological feature, each of them showed a unique pattern of distribution and/or phenology. Further examinations on *Ulva prolifera* that were most abundant and widespread in Mikata-goko were performed using the ribosomal ITS regions with much higher evolutionary rate than *rbc*L. A total of three ribotypes (Ⅰ, Ⅱ, and Ⅲ) and two heterozygotic ribotypes (Ⅰ/Ⅱ and Ⅰ/Ⅲ) were detected from autumn to spring, and no *U. prolifera* was collected in summer. While ribotypes Ⅰ and Ⅰ/Ⅱ were detected from all sites, the other two ribotypes were collected only from low-brackish (mean salinity < 5 psu) or high-brackish waters (> 13 psu), suggesting physiological differentiation among these ribotypes.

2.1.2 Identification of marine macroalgae using PCR-RFLP and DGGE methods, by Dr. Takeaki Hanyuda, Kobe University

PCR-RFLP method is effective for identification of algae species. DGGE method seems to be suitable for identification of algae species, especially detection of introduced species is expected.

2.1.3 DNA barcoding of macroalgae – 'Hawaiian Rhodophyta Biodiversity Project' as a case study of DNA-based floristic surveys -, by Dr. Akira Kurihara, Kobe University

DNA barcoding was embarked on establishing a substantial database of mitochondrial cytochrome c oxidase subunit Ⅰ (*cox* Ⅰ or CO Ⅰ) gene sequences, as DNA tags, with the objective of facilitating the rapid identification of any biological specimen of animals. Subsequently, its concept has been expanded over various organisms, and the DNA barcoding is now used as a preliminary screening tool for understanding of species diversity, which would be applicable to the following ways: early detection of alien species introductions, monitoring of invasive species, molecular discrimination among native and invasive species in question. In my talk, I will talk about (i) the concept and workflow of DNA barcoding and (ii) Hawaiian Rhodophyta Biodiversity Project as a case study of DNA-based macroalgal floristic surveys, on which I have been worked during my time in Alison Sherwood Laboratory, University of Hawaii, in 2006-2010.

2.1.4 Single-cell PCR technique and its application to phylogenetic studies, by Prof. Takeo Horiguchi, Hokkaido University

In this lecture, I would like to talk about the techniques which we routinely use for the systematic studies of microalgae. Although my main topics deal with microalgae, this technique can be applicable to the systematic studies of macro-algae as well.

For systematic study of microalgae, we need several different types of data, i.e. light micrographs, scanning electron micrographs (SEM), transmission electron micrographs (TEM), and molecular data. To obtain these data, we usually culture the organisms first. However, one big problem is that many of the microalgae and protists are not always easy to culture. Since, having cultures is important and thus we should try to obtain cultures of target organisms as far as possible. However, if it is not possible, we need to seek other ways. One possible solution is the method which I am going to introduce here, i.e. single-cell PCR technique coupled with morphological observations.

This technique involves morphological observations using light microscope (LM), scanning electron microscope and single-cell PCR. Before a@@lying the cells to single-cell PCR, it is extremely important to retain morphological record by taking photographs of high quality (LM and/or SEM), because for microalgae, it is not possible for retain voucher specimens. In this talk, I would like to introduce a successful example of this technique.

A heterotrophic marine dinoflagellate *Protoperidinium oblongum* produces several different types of motile cysts. Based on the fact that these different types of cysts produce similar type of motile cells, Wall and Dale (1968) concluded that these morphological differences in cysts are actually intra-specific variation. We have re-examined *P. oblongum* using the technique mention above (Fig. 1). We collected cysts of *P.oblongum* from Lake Saroma, Hokkaido and cysts were subjected to germination experiment. We found two different types of cysts, i.e. Type 1 and Type 2. We then observed germinated motile cells in detail and found that the motile cells from different types of cysts are different from each other in the shape of 2a plate (Fig.2). After observing the morphology, each cell was subjected to single-cell PCR and SSU rDNA were sequenced. It was demonstratedthat these two types are also genetically distinguishable from each other (Fig.3). We therefore, concluded that two different types of cysts represent species difference.

The single-cell PCR technique can be applied to macroalgal studies. When extracting DNA from seaweed tissue is difficult, the use of single-cell PCR method using unicellular stages-monospores, zoospores, eggs and sperms, can be useful. Recently, we used FFPE kit (Qiagen) for single-cell PCR. This kit was designed to extract DNAs from formalin-fixed and paraffin-embedded tissues. We found that use of this kit increased success rate of single-cell PCR and of course, this can be applicable to seaweed samples.

(see the Appendix 5 about Power point Slides of **2.1.1** to **2.1.4**)

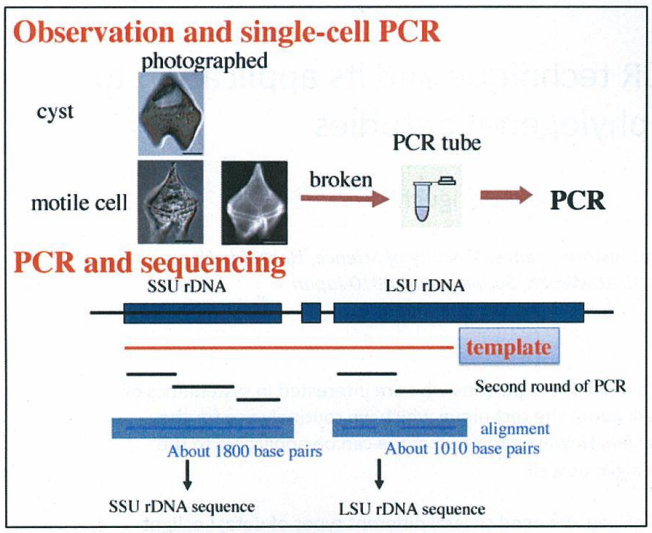


Figure 1. Outline of methods of morphological observations coupled with single-cell PCR technique.

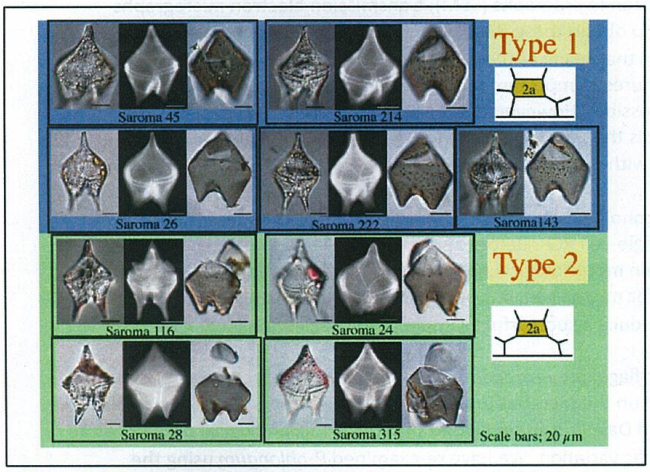


Figure 2. We have found two different types of cysts in Protoperidinium oblongum-complex. These two types produce very similar motile cells. However, these motile cells can be distinguished by the shape of 2a plate.

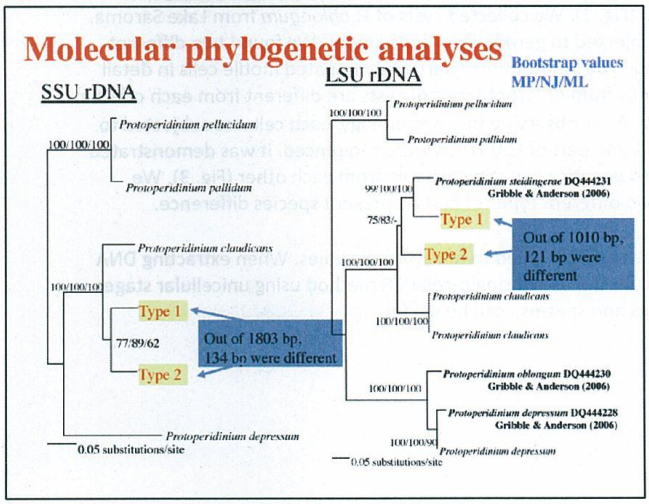


Figure 3. Molecular phylogenetic studies based on single-cell PCR technique. It is evident that two morphotypes can be also distinguished genetically.

2.2 Individual training practice

Training for mastering basic skills in identification using genetic marker from its basic to application was performed for five days with one-on-one hands-on training in three institutions: Kobe University,

Hokkaido University and Fukui Prefecture University.
As an example of the individual training practice for five days, the practice at Hokkaido University was shown in detail as below.

The first day: Extracting DNA, Purifying DNA

- Preparation of samples for DNA extraction
  1) Samples dried in silica gel.
  2) Samples preserved in ethanol.
  3) Frozen samples
  4) Pressed specimens

  Samples of 1)-3) are recommended for DNA extraction. It is difficult to extract a PCR-grade DNA from pressed specimens in many cases. Formalin-preserved specimens are unsuited for DNA analyses.
- Extracting DNA

  There are a lot of methods for extracting DNA from organismal tissues. For algae and plants, major methods are to use CTAB or a DNeasy Plant Mini Kit (QIAGEN Inc., Valencia, CA, USA). The following method is modified in our laboratory from some methods and simple and low-cost.

  1. Place a fragment (25 mm^2) of a thallus in a 1.5 ml microfuge tube.
  2. Add 150 µl of YK buffer and 8 µl 2-mercaptoethanol.
  3. Grind the fragment, using a hand-operated motor with a plastic pestle.
  4. Add 400µl of YK buffer*[1], and centrifuge for 2 sec, and transfer the supernatant to a fresh tube.
  5. Centrifuge the tubes into which the supernatant was transferred at 10000 rpm for 5 min.
  6. Discard the supernatant, and add 800 µl of YK buffer and 8 µl 2-mercaptoethanol, and suspend the pellet, shaking the tube. Incubate in a water bath at 50˚C for 10 min.
  7. Centrifuge at 10000 rpm for 5 min.
  8. Discard the supernatant, and add 450 µl of HTE buffer*[2], 8 µl of 2-mercaptoethanol, 120 µl of 8M urea and 150 µl of chloroform, and suspend the pellet, shaking the tube. Mix gently for 20 min at room temperature.
  9. Add 270 µl of 3M sodium acetate and mix, and place on ice for 10 min.
  10. Centrifuge at 12000 rpm for 10 min at 4˚C.
  11. Transfer 540 µl of the supernatant to a fresh tube, and add 450 µl of isopropanol and mix gently.
  12. Centrifuge at 12000 rpm for 10 min.
  13. Discard the supernatant, and add 800 µl of 70% ethanol and mix gently.
  14. Discard the supernatant, and suspend in 100 µl of sterilized distilled water.
  15. Freeze or apply to purification with Standard Super-Cel.

  CAUTION: Chloroform is harmful to both human health and the environment. It has to be trashed into a designated waste bottle.

  *[1] YK buffer
  10 mM Tris-HCl, 10 mM Na_2EDTA, 1 mM spermidine, 10 mM NaCl
  Adjust pH to 9.4 by 10N NaOH

  *[2] HTE buffer
  50 mM Tris-HCl, 20 mM Na_2EDTA
  Adjust pH to 7.5 by 10N NaOH
- DNA purification with Standard Super-Cel
  1. Add 300 µl of 6M NaI to a tube containing 100 µl DNA solution.
  2. Add 15 µl of Standard Super-Cel suspension*[3] and mix gently and incubate at room temperature for 5 min. (DNA binds to the silica)
  3. Centrifuge in a microcentrifuge for 30 sec.
  4. Discard the supernatant, and add 500 µl of NEW Wash*[4], suspend the pellet, shaking the tube.

5. Centrifuge in a microcentrifuge for 30 sec, and discard the supernatant.
6. Wash the pellet 3 times with NEW Wash, repeating the steps 4 and 5.
7. Add 60 µl of sterile distilled water and suspend the pellet. (DNA elutes in water)
8. Centrifuge in a microcentrifuge for 30 sec.
9. Transfer 50 µl of the supernatant to a fresh tube.
10. Add 50 µl of sterile distilled water to the tube including Standard Super-Cel and suspend the pellet. Centrifuge the tubes for 30 sec.
11. Transfer 50 µl of the supernatant to the tube to which the first eluted DNA is transferred.
12. Freeze until required.

This cleaned DNA can be used as template for PCR.

*[3] Standard Super-Cel (Nakalai Tesque, Inc., Kyoto, Japan) suspension
Put the powder of Standard Super-Cel into a fresh tube up to ca 500 µl, and add 1000 µl of sterilized distilled water to wash the powder. Spin down the powder and discard the supernatant. After 3 times of washing, add 800 ml of sterilized distilled water. Use just after suspending. It should be preserved by freezing.

*[4] NEW Wash
50% EtOH, 0.1M NaCl, 10mM Tris-HCl, 1mM EDTA
pH 7.5

The second day: PCR, Electrophoresis.

- Prepare PCR reaction mixture.

Takara ExTaq (5 units/ml) 0.12 µl, 10x Buffer 2.5 µl, dNTP mixture (2.5 mM each) 2 µl, Primer1 (20 pmol/ml) 0.25-1 µl, Template DNA 0.5 µl
Add sterilized distilled water up to 25 µl.
Follow a manufacture's instruction. When amplifying ribosomal DNA, add 1.25 µl of DMSO (5% in final concentration).

- PCR condition

94˚C for 1 min
94˚C for 20 sec
55˚C for 20 sec } 35-50 cycles
72˚C for 45 sec
72˚C for 5min

PCR condition may change depending on Taq polymerase, primers, the length of amplified DNA and a thermal cycler.

- Hot start method

Run a thermal cycler and place the tubes in the equipment when the temperature becomes over 90˚C in the first denaturation. This method prevents miss annealing.

- Freezing

After thermal cycling is finished, freeze the tubes to preserve.

- Agarose gel electrophoresis

Agarose electrophoresis is performed to check PCR products.

1. Add 125 mg of agarose to a conical flask, and further add 12.5 ml of TAE buffer*[5], and swirl to mix.
2. Put the flask into a microwave, and heat on for 1 min. Swirl the flask. If all agarose is dissolved, heat again.
   CAUTION: The flask is hot; make sure to ware a glove.
3. Allow the mixture to cool for 3 min and prepare a gel tray.
4. Pour the mixture into the gel tray and set a comb.
5. Wait for 30 min to allow the gel to set.
6. Pour a little amount of TAE on the gel, and carefully remove the comb by pulling it upwards.
7. Place the gel with tray into an electrophoresis cell containing TAE buffer.

8. Cut a piece of parafilm® and place it on the bench top and scratch it to stick it on.
9. Place small dots (ca. 1µl) of loading buffer onto the parafilm® (one dot for each PCR sample).
10. Take 2.5 µl of a sample and mix it with the dot by pipetting up and down. Then, pipette up the mix and gently load it into the well of the gel. DNA size maker also may be loaded.
11. Put the cell cover on and turn on the power supply. Run the electrophoresis rig for ca. 15 min at 100 volts.
12. Turn off the power supply. Remove the gel and tray with hands gloved, and slide the gel off of the tray and put the gel into ethidium bromide solution.
13. Soak the gel in the ethidium bromide solution for 10 min. Using a spatula, transfer the gel onto plastic wrap.
14. Put the gel and wrap on a UV light box and turn on the UV light box.
15. If bands are visible, take a picture.
16. Transfer the gel and wrap into a reservoir containing bleach solution which decomposes ethidium bromide.

CAUTION: Ethidium bromide is a mutagen. Put on groves when you use the chemical.

*[5] TAE buffer

Tris 40 mM, Acetic acid 40 mM, Na_2EDTA 1 mM

The third day: PEG precipitation, Cycle sequencing

- PEG precipitation (Purification of PCR products)

1. Add 25 µl of sterilized distilled water to PCR products (22.5 µl).
2. Add 30 µl of PEG-NaCl solution*[6] and mix thoroughly with a vortex mixer.
3. Place tubes on ice for 1 hr.
4. Centrifuge at 14000 rpm for 10 min at 4℃.
5. Discard the supernatant carefully by pipetting. Make sure not to suck out the pellet, which is not visible.
6. Add 150 µl of 70% ethanol.
7. Centrifuge at 14000 rpm for 10 min at 4℃.
8. Remove the supernatant carefully and dry up.
9. Add 8 µl of sterilized distilled water to dissolve precipitation of DNA.
10. Preserve at -20℃

*[6] PEG-NaCl solution:

5g PEG (polyethylene glycol #6000) and 3.65g NaCl in 25ml H_2O

- Cycle sequencing

BigDye Terminator v1.1 Cycle Sequencing Kit (Applied Biosystems) is used in this course.

1. Prepare reaction mixtures of the following contents in each PCR tube. Practically prepare the reaction mixtures excluding DNA (and primers) and add DNA (and primers) to each tube after that.

1/4 Reaction Concentration and 1/2 Reaction Volume
(for sequencing DNA more than 500 bp)

	(µl)
Ready Reaction Premix (2.5 x)	1
Sequencing Buffer (5 x)	1.5
Water	1.5
DMSO	0.5
Primer (1 pmol/µl)	1.6
Template DNA Solution	3.9
Total Volume	10

1/8 Reaction Concentration and 1/2 Reaction Volume
(for sequencing DNA less than 500 bp)

	(µl)
Ready Reaction Premix (2.5 x)	0.5
Sequencing Buffer (5 x)	1.75
Water	1.75
DMSO	0.5
Primer (1pmol/µl)	1.6
Template DNA Solution	3.9
Total Volume	10

2. Set tubes in a thermal cycler and perform cycle sequencing reaction with the following condition.

Condition of cycle sequencing

96˚C, 1 min
96˚C, 10 sec
50˚C, 5 sec } 25 cycles
60˚C, 4 min
4˚C, hold

3. These products can be stored at -20˚C.

The fourth day: Purification, Sequencing

- Purifying cycle sequencing products

Ethanol/EDTA/Sodium Acetate Precipitation

1. To prepare the mixture, add followings to a fresh 1.5 ml tube and mix.

	Volume for one reaction tube
Water	10 µl
125mM EDTA	2 µl
3 M Sodium Acetate	2 µl

2. Add 14 µl of the mixture to each tube containing products.
3. Add 50 µl of 95% ethanol to each tube and mix.
4. Incubate at room temperature for 15 min.
5. Centrifuge the tubes at 15000 rpm for 20 min at 6˚C.
6. Discard the supernatant and add 150 µl of 70% ethanol.
7. Centrifuge the tubes at 6˚C and 15000 rpm for 5 min.
8. Discard the supernatant and dry up.
9. These products can be stored at -20˚C.

The fifth day: Phylogenetic analysis

- Phylogenetic analyses using PAUP

PAUP*[7] is one of popular phylogeneitic analysis program and infers phylogenetic trees using maximum parsimony, neighbor joining and maximum likelihood methods. Purchasing information of the PAUP can be found at http://paup.csit.fsu.edu.

*[7] PAUP uses the NEXUS format for input data files. An example of the NEXUS format is below.

```
#NEXUS
Begin data;
Dimensions ntax = 5 nchar = 10;
Format data type = nucleotide gap = - missing = N;
Matrix
A001   ATGCTTACGATC
A002   ATCCTT-CGATC
B001   ATCGTAACGATC
```

B002 ATGCTAACGATC
C001 ATAATTACGTTN
;
END;

- Maximum parsimony analysis by PAUP.
  1. Run PAUP and execute a file. PAUP uses the NEXUS format for input data files.
  2. Type "outgroup *** *** (OTU label)" to specify taxa as an outgroup.
  3. Type "BandB" in the command line and run: branch and bound search is performed.
  4. Type "showtree 1" to show the inferred tree.
  5. Type "savetrees file=*.tre brlen=yes from=1 to=1" to save the tree with branch lengths.
  6. Type "bootstrap nrep=100 search=heuristic" to perform bootstrap analysis.
  7. Type "savetrees file=*boot.tre savebootp=nodelabels from=1 to=1
  8. Run TreeView program and open the saved tree file. Print trees.

(see the Appendix 6 about all textbooks used for training practice)

2.3 Summary workshop

The summary workshop was held to exchange the results of training of all trainees and comments together with instructors, and to take a lecture from invited commentator or project leader.

2.3.1 The results of training of all trainees

2010

Han XIAOTIAN (CHINA)
"The Study of Molecular Identification of *Ulva* sp. from Chinese Sea Based on Internal Transcribed Spacer Sequences Analysis"

Minhthanh Thi NGUYEN (VIETNAM)
"Molecular Taxonomy of Marine Macroalgae"

Anchana PRATHEP (Thailand)
"the *Halimeda* Systematic Study"

Woan-Shien NG (MALAYSIA)
"Morphological Characteristics with Phylogenetic Analysis and Comprehensive Phylogenetic Analysis on the Malaysian *Sargassum* Species Using Sequences of RuBisCo Gene"

Soradkorn PHIMRA (THAILAND)
"Macroalgal Taxonomy Using Genetic Markers: A Case Study on Genus *Laurencia* Complex"

Roike Iwan MONTOLALU (Indonesia)
"Macroalgal Taxonomy Using Molecular Markers: In Case Study *Kappaphycus*"

2011

Tsz Yan NG (CHINA)
"To Learn the Knowledge and Techniques on Molecular Taxonomy of Marine Macroalgae"

Phara CHEA (CAMBODIA)
"Taxonomy of Marine Macroalgae Using Molecular Markers"

Ngocmai Thi DINH (VIETNAM)
"Application of Molecular Marker Techniques in Taxonomic Identification of a *Ulva* Species Collected from Obama Bay"

Phaik Eem Lim (MALAYSIA)
"Genetic Diversity of *Kappaphycus alvarezii* and Species Identification of *Kappaphycus* and *Eucheuma* using SSCP Aproach"

Narongrit MUANGMAI (THAILAND)
"Molecular Tool Rapid Identification and Effective Evidence in Taxonomic Studies: Case Study; Identify of a Small Flattened Species of *Gracilaria (*Gracilariaceae) Collected from Andaman Sea, Thailand"

Sze-wan POONG (MALAYSIA)
"DNA extraction on the samples of *Mesospora schmidtii*, my own dried sample using conventional DNA extraction which use a low cost modified method"
(in presentation order)
(see the Appendix 7 about Power Point Slides of training results of all trainees)

2.3.2 Lecture from Invited Commentator/Project Leader
2010
Invited commentator, Prof. Anong CHIRAPART (Algal Bioresources Research Center, Department of Fishery Biology, Faculty of Fisheries, Kasetsart University, Thailand)
"Status Macroalgal Taxonomy in Southeast Asia, and Problems for Fishery, Environmental Study and Biodiversity in Thailand.
2011
Project leader, Prof. Hiroshi KAWAI ((Kobe University Research Center for Inland Seas)
"Introduction (?) of marine organisms"
(see the Appendix 8 about Power Point Slides of two lectures)

3.0 Results and Discussion

Trainees were informed of the aim and purpose of the program through orientation and explanation. They learned the processes of phycology taxonomy analysis techniques by individual training practice for six days. Training program included: (1) DNA extraction, (2) purification of DNA, (3) agarose gel electrophoresis, (4) PEG precipitation, cycle sequencing, (5) electrophoresis, (6) phylogenetic analysis, (7) PCR technique and etc.. On the summary meeting they exchanged their practical results and comments to practices with instructors and each other.

The training schedule was quite limited, but all trainees got the new knowledge and skills. Furthermore, this training provided the opportunity for dialogue with other participants. The network in this field will be built in the near future.

4.0 Conclusions

This project aimed to provide practical trainings of investigation techniques for the molecular phylogemetic analysis to the researchers of Southeast Asia. Furthermore, we aimed to establish regional network of researchers for developing research collaborations in the study of non-indigenous marine algae, because exchange of taxonomic and genetic information of local populations in the adjacent areas is specially important in studying non-indigenous taxa.

This project, although the period of preparation as well as the actual practice courses was rather short, we believe the program was productive, and we enjoyed the communications among all participants. We hope that the good friendship and academic relationship established during the program will continue, and help to strengthen the network.

5.0 Future Directions

The study related with non-indigenous organisms, it is essential to have basic taxonomic information (inventory study of the local fauna and flora), and also the information of other areas including the donor areas of them. Therefore, the establishment of taxonomic studies in the area is not sufficient, and it is important to establish international networks to share the biodiversity information. In the present program, we communicated with many researchers in the target countries, but found difficulties in obtaining information of the research status, because of the lack of such networks of researchers. Therefore, we hope to keep frequent communications with the trainees and to establish tight relationships with them, and help them to strengthen the local networks through such programs.

References

Wall D, Dale B (1968). Modern Dinoflagellate Cysts and Evolution of the Peridiniales. *Micropaleontol*. **14**: 265-304.

Appendix 1 Program

2010 Program

CAPaBLE Training Program

Capacity Building of Biodiversity Research in Coastal Zones

of the Asia Pacific Region

Macroalgal Taxonomy Using Genetic Markers

3-13 July 2010

Kobe, Obama, Sapporo

PROGRAM

3 July (Sat), 2010: Arrival to Kobe University

4 July (Sun): Introduction meeting [Venue: Z-201, Faculty of Science, Kobe University]
10:00 Welcome remarks
Linda Anne STEVENSON (APN Center)
Masakazu FURUKAWA (International EMECS Center)
Hiroshi KAWAI (Kobe University Research Center for Inland Seas)
10:30 Introduction of the participants
11:00 Guidance of the programs (H. Kawai, EMECS)
12:00 Lunch
13:00 Lectures on taxonomic study of macroalgae using molecular markers and morphology
M. Kamiya: Taxonomy of marine macroalgae using molecular markers.
T. Hanyuda: Identification of marine macroalgae using DGGE and PCR-RFLP methods.
A. Kurihara: DNA barcoding of marcolagae.
T. Horiguchi: Single-cell PCR technique and its application to phylogenetic studies.
15:00 Closing remarks and departure to host laboratories

5 (Mon) -10 (Sat) July: Practical training in each host laboratory (Kobe University, Hokkaido University, Fukui Prefectural University)

11 July (Sun): Travel to Kobe University

12 July (Mon): Summary meeting. [Venue: Takigawa Memorial Hall, Kobe University]
10:00 Report from each trainee on the achievement and perspective of future research program (All participants)
12:00 Lunch
13:00 Comments from instructors and invited commentator (A. Chirapart)
14:00 General discussions
15:00 Closing remarks (APN, EMECS, KURCIS)
17:00 Farewell Reception and exchange with some of the PICES Rapid Assessment Demonstration Workshop [Venue: Takigawa Memorial Hall, Kobe University]

13 July (Tue): Departure of participants

List of Participants

Trainees

Dr. Xiaotian HAN
Institute of Oceanology, Chinese Academy of Science, China
Email: xthan@qdio.ac.cn

Dr. Roike Iwan MONTOALU
Sam Ratulangi University, Indonesia
Email: rmontolalu@yahoo.com

Ms. Minhthanh Thi NGUYEN
Institute of Biotechnology, Vietnamese Academy of Science and Technology, Vietnam
Email: mthanh126@yahoo.com

Ms. Soradakorn PHIMLA
Casetsart University, Thailand
Email: soradakorm_3@hotmail.com

Dr. Anchana PRATHEP
Prince of Songkla Unviersity, HatYai, Thailand
Email: a_prathep@hotmail.com

Ms. Ng Woan SHIEN
University of Malaya, Malaysia
Email: woanshien@yahoo.com

Invited Commentator

Dr. Anong CHIRAPART
Casetsart University, Thailand
Email: ffisanc@ku.ac.th

Instructors

Dr. Takeaki HANYUDA
Kobe University Research Center for Inland Seas
Email: hanyut@kobe-u.ac.jp

Dr. Takeo HORIGUCHI
Department of Natural History Sciences, Faculty of Science, Hokkaido University
Email: horig@mail.sci.hokudai.ac.jp

Dr. Mitsunobu KAMIYA
Faculty of Marine Bioscience, Fukui Prefectural University
Email: mkamiya@fpu.ac.jp

Dr. Hiroshi KAWAI
Kobe University Research Center for Inland Seas

Email: kawai@kobe-u.ac.jp

Dr. Kazuhiro KOGAME (Hokkaido University)
Department of Natural History Sciences, Faculty of Science, Hokkaido University
Email: kogame@sci.hokudai.ac.jp

Dr. Akira KURIHARA (Kobe University)
Kobe University Research Center for Inland Seas
Email: akirak@harbor.kobe-u.ac.jp

Organizer

APN Center (info@apn-gcr.org; http://www.apn.gr.jp)

Mr. Tetsuro FUJITSUKA

Dr. Linda Anne STEVENSON (APN Center)

International EMECS Center (secret@emecs.or.jp; http://www.emecs.or.jp)

Mr. Kazuo ARASHI (International EMECS Center)

Mr. Masakazu FURUKAWA (International EMECS Center)

Mr. Eiji ISHIHARA (International EMECS Center)

Dr. Satoshi UMEMOTO (International EMECS Center)

Ms. Ayako YAMADA (International EMECS Center)

Other information

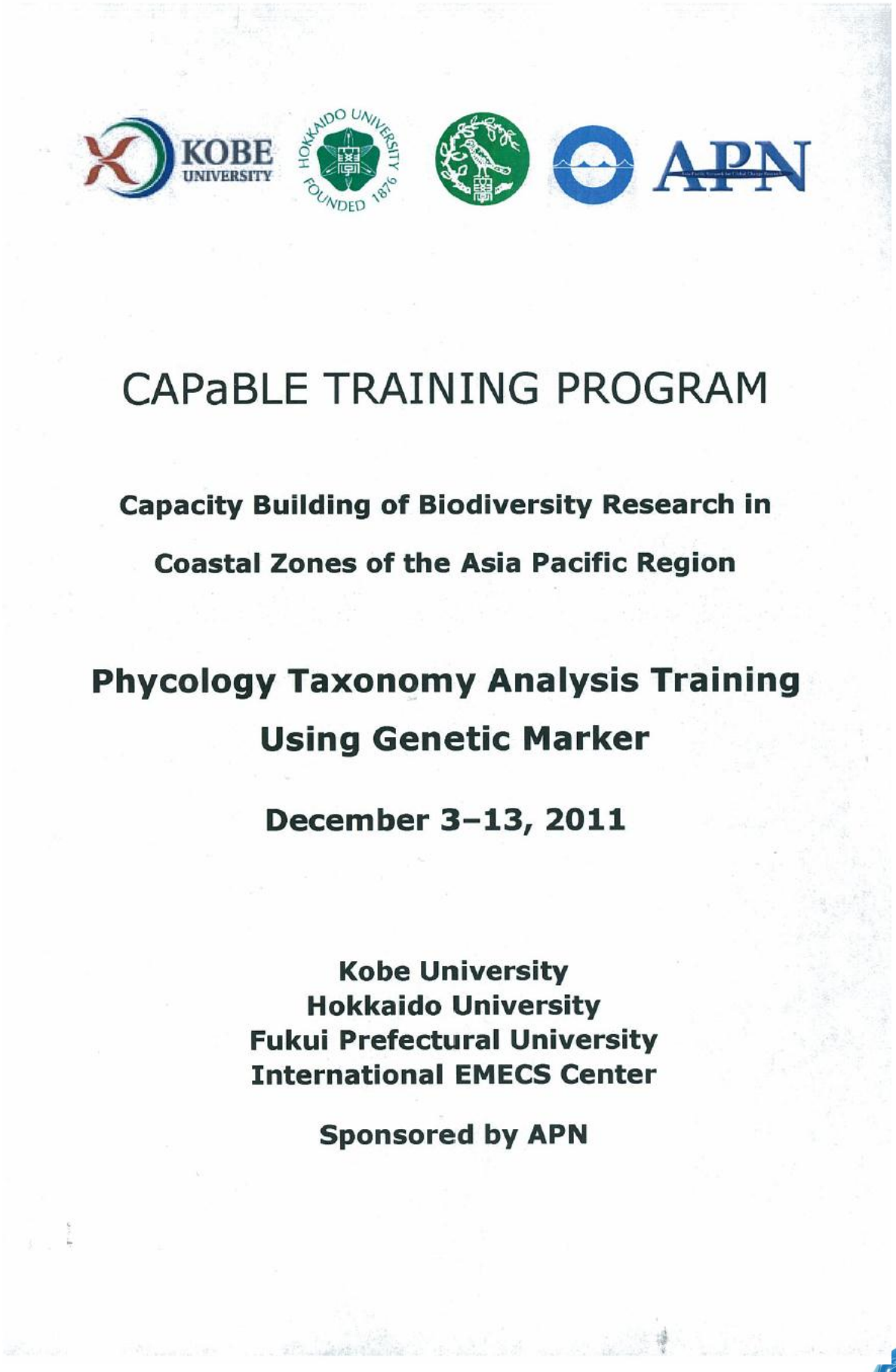
Venue of introduction and summary meetings
Faculty of Science Building and Takigawa Memorial Hall, Kobe University

Host Laboratories
1) Kobe University Research Center for Inland Seas (Faculty of Science Building)
1-1 Rokkodai, Nadaku, Kobe, 657-0013 Japan.
Phone. +81-78-803-5781; Fax: +81-78-803-6698
Anchana PRATHEP and Woan-Shien NG

2) Department of Natural History Sciences, Faculty of Science, Hokkaido University
Sapporo, 060-0810 Japan.
Phone: +81-11-706-2738; Fax:+81-11-706-4851
Xiaotian HAN and Minhthanh Thi NGUYEN

3) Faculty of Marine Bioscience, Fukui Prefectural University
1-1 Gakuencho, Obama, Fukui, 917-0003 Japan.
Phone & Fax: +81-770-52-9606
Soradkorn PHIMLA and Roike Iwan MONTOLALU

2011 Program



CAPaBLE TRAINING PROGRAM

Capacity Building of Biodiversity Research in

Coastal Zones of the Asia Pacific Region

Phycology Taxonomy Analysis Training Using Genetic Marker

December 3–13, 2011

Kobe University
Hokkaido University
Fukui Prefectural University
International EMECS Center

Sponsored by APN

Agenda

3 Dec (Sat), 2011: Arrival to Kobe University

'MK Skygate Shuttle Omnibus Service' from Kansai Airport to KURCIS Rokkodai Campus (Faculty of Science Building) (http://www.mk-group.co.jp/english/shuttle/index.html) will be arranged by the organizer. After registration, the trainees will check-in to the Kobe University Guesthouse 'Gakujiso' in the afternoon.

4 Dec. (Sun): Introduction meeting [Venue: Kobe University]

10:00 Welcome remarks (EMECS, KURCIS)

10:10 Introduction of the participants (All participants)

10:30 Guidance of the programs (H. Kawai, EMECS)

11:00 Lectures on taxonomic study of macroalgae using molecular markers and morphology

(M. Kamiya: Taxonomy of marine macroalgae using molecular markers.)

(T. Hanyuda: Identification of marine macroalgae using SSCP and DGGE methods.)

12:30 Lunch

13:30 Lectures on taxonomic study of macroalgae using molecular markers and morphology

(A. Kurihara: DNA barcoding of marcolagae.)

(T. Horiguchi: Single-cell PCR technique and its application to phylogenetic studies.)

15:00 Closing remarks and departure to host laboratories

5-9 Dec: Practical training in each host laboratory (Kobe University, Hokkaido University, Fukui Prefectural University)

10 Dec. (Sat): Travel to JICA Hyogo

11 Dec. (Sun): Summary meeting. [Venue: Kobe University]

10:00 Report from each trainee on the achievement and perspective of future research program (All participants)

12:00 Lunch

13:00 General discussions

14:00 Closing remarks (APN, EMECS, KURCIS)

15:00 Farewell Reception [Venue: Kobe University]

12 Dec. (Mon): Preparation of program report. [Venue: JICA Hyogo]

13 July (Tue): Departure of participants. 'MK Skygate Shuttle Omnibus Service' from JICA Hyogo.

Accommodation

In Kobe, the participants will stay in the Kobe University Guesthouse 'Gakujiso' near JR Rokkomichi Station and JICA Hyogo.

Host Laboratories

1) Kobe University Research Center for Inland Seas (Faculty of Science Building)
1-1 Rokkodai, Nadaku, Kobe, 657-8501 Japan.
Phone. +81-78-803-5781; Fax: +81-78-803-6698
Phaik Eem LIM (Malaysia), Narongrit MUANGMAI (Thailand)

2) Department of Natural History Sciences, Faculty of Science, Hokkaido University
Sapporo, 060-0810 Japan.
Phone: +81-11-706-2738; Fax:+81-11-706-4851
Tsz Yan NG (China), Sze-Wan POONG (Malaysia)

3) Faculty of Marine Bioscience, Fukui Prefectural University
1-1 Gakuencho, Obama, Fukui, 917-0003 Japan.
Phone & Fax: +81-770-52-9606
Ngocmai Thi DINH (Vietnam), Phala CHEA (Cambodia)

Appendix 2 List of Young Scientists and Their Message extracted from training reports

2010 Trainees

Han XIAOTIAN (China) (Associate Professor, Institute of Oceanology, Chinese Academy of Science, tianyan@qdio.ac.cn).

This training program was very considerate for every trainees with differently professional background. The knowledge of molecular identification and experiences were very effective and actual in the short time stayed in Japan.

Roike Iwan MONTOLALU (Indonesia) (Lecturer/Researcher, Fisheries and Marine Science, Sam Ratulangi University, montolalu@yahoo.com).

Woan-Shien NG (Malaysia) (PhD student, Institute of Biological Sciences, Institute of Ocean and Earth Sciences, University of Malaya, woanshien@yahoo.com).

This training program provided a very good approach to train and educate new knowledge to the representative from each country. During the training, we did exchange new information and collaboration among the participants. The knowledge we obtained is important for us to pass on to new students as well as build up the next generation of seaweed taxonomists for the university and country.

Although the training was short in terms of obtaining a good results to data analysis, the basic knowledge on the molecular were pass on due to one-on-one intensive daily training. I would suggest that an extension of the period of training to a month for a more complete training on the data analysis. I believed that data analysis and tree interpretations are crucial for producing a better discussion on the results obtained.

Anchana PRATHEP (Thailand) (Assistance Professor, Department of Biology, Faculty of Science, Prince of Songkla Unviersity, a_prathep@hotmail.com).

Although, the timing is rather short but it still allowed us to have a grasp at all aspects and understand the molecular technique as a whole. The venue and facilities are great and the communication before coming to the workshop with the instructor is very useful and it helps the participants to prepare themselves or asks some questions if needed. The instructors are friendly and helpful; and also with the experience and authority, they could have certainly guide us for further work, which is rather important and useful.

I really do have a great time and look forward for further collaborations; and this programme is very useful and would be benefit for the region.

Soradkorn PHIMLA (Thailand) (Researcher, Algal Bioresorces Research Center, Department of Fishery Biology, Faculty of Fisheries, Kasetsart University, saradakorn_3@hotmail.com).

I have grateful opportunity to participate in “Capacity Building of Biodiversity Research in the Coastal Zones of the Asia Pacific Region: Phycology Taxonomy Analysis Training Using Genetic marker (APN CAPaBLE Program)” at Kobe University and Fukui Prefecture University, Japan from 4- 12 July 2010. The training program give a lot of useful and completely respond for working as researcher at ABRC. I hope to receive accurate analysis skill for seaweed identification using molecular marker when I come back to Thailand.

Minhthanh Thi NGUYEN (Vietnam) (Researcher, Algal Biotechnology Department, Institute of Biotechnology, Vietnamese Academy of Science and Technology, mthanh126@yahoo.com).

This training program is very useful for my researches because in Vietnam there are not enough experts to help our laboratory in phylogenetic taxonomy. I have learned many value experiences about genetic marker and application in phylogenetic taxonomy. In addition, I met many experts and young researchers from other countries who are all concern about phylogenetic taxonomy. After this training program. I will try my best to apply these lessons in my researching and for further studies in my laboratory and in my country.

2011 Trainees

Phala CHEA (Cambodia) (Acting Chief, Genetic and Fish Disease Division, Marine Aquaculture Research and Development Center, cheaphala@yahoo.com).

Taxonomy of marine macroalgae using molecular markers is a new technology and very convenience for scientist to compare among phylogenetic distant organisms, various genetic information, stable against environmental and developmental variation, easy to define theoretical models and apply statistical analyses, and possible to estimate divergence time. In my opinion, this training was very important for participants that provided not only new knowledge but also gave opportunity to create strong networking amongst participants and countries. As the first attempt in promoting a network based on molecular analysis in a way that is complementary to the traditional taxonomy group, the training was carried out very successfully. The knowledge we obtained is important for us, especially for country poor information and limit in biodiversity research like Cambodia.

Tsz Yan NG (China) (PhD Student/ Graduate Demonstrator, Marine Science laboratory, School of Life Sciences, The Chinese University of Hong Kong, ngtszyan2011@gmail.com).

I am very satisfied with the whole training program. With some basic knowledge of molecular techniques, I find the training contents very useful in improving my techniques. Among the training contents, cycle sequencing and sequencing with a genetic analyzer are fully new to me. After getting familiar with the working principle through the training, undoubtedly I will be able to get better results when using the same techniques again. Moreover, I think the training is very practical.

Furthermore, I believe the program has successfully helped to build up a network among researchers for information exchange. Trainees meet and get on well with each other throughout the training program. So it should be possible for us to share taxonomical information in the near future.

Phaik Eem LIM (Malaysia) (Coordinator for Molecular Biology Section/Senior Lecturer, Algae Research Laboratory, Institute of Ocean and Earth Science, University of Malaya, phaikeem@um.edu.my).

This training has allowed me to learn a new molecular DNA fingerprinting method - single strand conformation polymorphism (SCCP) which will be very useful for my future research. In addition to this, it also gives us the opportunity to foster the networking between South East Asia participants and Japanese researchers.

Even though the hand on training was conducted in a very short period of time (5 days) but early and active communication via email with my hosts in discussing the training objectives has helped me to achieve much in this training course.

In summary, I have learned a lot and gained valuable molecular technique for taxonomy purposes through this CAPaBLE training program.

Sze-wan POONG (Malaysia) (PhD Student, Algae Research Laboratory, Institute of Ocean and Earth Science, University of Malaya, c-one218@hotmail.com).

My overall opinion about this training program is that it represents a good platform for each participant to gain new knowledge and skills and in my case, put into perspective what we have learnt in theory. I have to admit that the practical training period of five days was rather short and a longer period of time may prove to be a more fruitful experience. Nonetheless, the program serves as a good opportunity for young researchers like us to build up our network among various countries. I believe that each and every one of us would be most happy and delighted to share the knowledge that we have picked up from the training program with our fellow colleagues in our laboratories or research centers.

Narongrit MUANGMAI (Thailand) (Research Assistant, Department of Fishery Biology, Faculty of Fisheries, Kasetsart University, seaweed_53@hotmail.com).

This workshop gives me a window of opportunity to create a dialogue with other

participants. We are from different places, different community and different culture, so when we come together we have a lot of things to talk and share.

Although the time for training is quite limited, but I am very confident that I get all that I expect to learn before coming here. No matter how long this training course I strongly believe that all instructors try their utmost best to teach and train all of us.

Ngocmai Thi DINH (Vietnam) (Researcher, Algal Biotechnology Department, Institute of Biotechnology, Vietnam Academy and Science and Technology, ddhong60vn@yahoo.com).

I think the training was carried out very successfully. Training program aimed to facilitate taxonomical information exchange among researchs and to conduct rapid assessments with a newly developed skill of taxonomy identification with genetic marker. After the training program, I will share the skills and information with other researchers and apply the basic identificatioon skill of genetic marker in the analysis of micro and macroalgae and other organism. I would like to suggest an extension of the period of training to one month, so as to achieve more complete training in data analysis.

(see the Appendix 3 about all of Training Report from trainees)

Appendix 3 Training Report from trainees

2010

- Han XIAOTIAN (China)

CAPaBLE Training Report

Han Xiaotian, Institute of Oceanology, Chiese Academy of Sciences, Qingdao, China, 266071

1) Myself introduction and background

Han Xiaotian, Female, 35years old,

Aassociate professor, doctor.

Institute of Oceanology, Chinese Academy of Science.

Research topic:

What I focused on were algae morphological taxonomy, molecular phylogeny and interspecific interaction. Now some researches about succession mechanism of macroalgae and microalgae are pursued and this is the content of projects as follows:

1. Key Project of Chinese National Programs for Fundamental Research and Development (973 program) (Grant No. 2010CB428700). "Mechanism of China's coastal algal bloom disasters and their biosecurity implications";

(This project were confirmed because of frequent occurrences of Enteromorpha bloom and microalgae bloom along Chinese sea in recent years)

2. Project supported by the Funds for Creative Research Groups of China (Grant No. 40821004). "Process and mechanism of ecological system succession of China's typical sea".

Experience

1. 2009-present, Associate professor, Institute of Oceanology, Chinese Academy of Science;
2. 2001-2009, Research Associate, Institute of Oceanology, Chinese Academy of Science;
3. 1998-2001, Research Assistant, Institute of Oceanology, Chinese Academy of Science.

Education

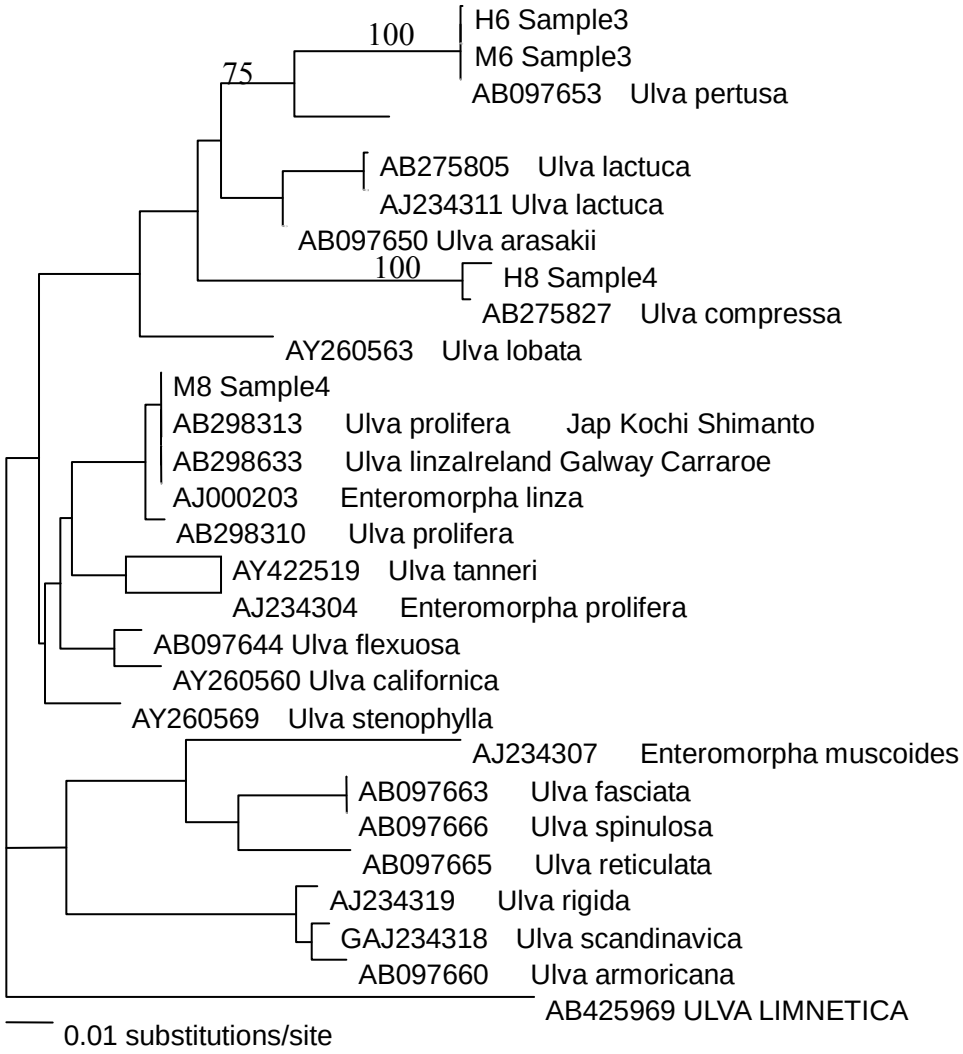
1. 2008-present, Doctor candidate, Marine biology, Ocean University of China;
2. 2001-2004, Master, Marine biology, Institute of Oceanology, Chinese Academy of Sciences
3. 1994-1998, Bachelor, Environmental sciences, Ocean University of China;

2) Training Summary

In the first day we take part in the training course from Hiroshi Kawai, Takeo Horiguchi, Mitsunobu Kamiya, Akira Kurihara, Takeaki Hanyuda. The instructors introduce the taxonomy of marine macroalgae using molecular markers, PCR-RFLP, DGGE, DNA barcoding, single-cell PCR technique and phylogeny analysis. The trainees can get the knowledge of molecular technique used in taxonomy of macroalgae.

From July 4 to 10, we experienced DNA extraction, purification, PCR amplification, sequencing technique directed by Takeo Horiguchi and Kazuhiro Kogame in Hokkaido University. Single-cell isolation and pretreatment of single-cell PCR also experienced in the laboratory. We get two *Ulva* species' sequence, then align sequences, construct Neighbor-joining tree and analyze the result. Four *Ulva* samples were detected, sample 1 and 2 has no PCR products because of they were old samples or lost during the experimental operation. ITS genes of Sample 3 and sample 4 were sequenced and analyzed in the neighbor-joining tree. Sample 3 was clustaled in one clade with *Ulva pertusa*, and samle 4 was clustaled in one clade with *Ulva compressa*. The bootstrap values were all 100.

After *Ulva* gene sequencing experiment, the single cell isolation was tried by myself under the light microscope. The details were showed by Professor Takeo Horiguchi. Also, pretreatment of the single cell PCR was showed to the trainees. Through these experiments and experience, we can realize molecular identification of macroalgae and microalgae.



Fig, 1 Neighbor-joining tree constructed from ITS sequences of *Ulva* species

3) Your opinion about the training in general

This training program was very considerate for every trainees with differently professional background. The knowledge of molecular identification and experiences were very effective and actual in the short time stayed in Japan.

- Roike Iwan MONTOLALU (Indonesia)

- Woan-Shien NG (Malaysia)

CAPaBLE Training Report

Capacity Building of Biodiversity Research in the Coastal Zones of the Asia Pacific Region: Phycology Taxonomy Analysis Training Using Genetic Marker

From July 3 to July 13, 2010

By NG WOAN SHIEN (Malaysia)

CONTENTS

1. Introduction and background of myself

I graduated from University of Malaya, Malaysia as Bachelor of Science (Honours) majoring in Genetics and Molecular Biology in year 2005. I then registered as a Master of Science student, working on the molecular studies of *Sargassum* species in Malaysia. In year 2007, I successfully converted from Master to PhD, majoring in Algal Biotechnology. I am currently under the supervision

of Prof. Dr. Phang Siew Moi in the University of Malaya. As for my PhD project, I work on the morphological and molecular taxonomy of *Sargassum* species in Malaysia and Indo-Pacific Region. As a member of SEASTax, I had been involved in the general work of morphological taxonomy of species from Malaysia. A combination usage of both morphological and molecular approaches has always been important for my routine work frame.

2. Training Summary

Sargassum is my taxa of interest for this training program. It is the most morphologically differentiated and complex genus in *Phaeophyceae*. There are about 400 valid species among 845 species, variety and form recorded in Algaebase. There are 39 species reported from Malaysia, with 25 species identified from the University of Malaya Seaweed and Seagrasses Herbarium. There is only subgenus *Sargassum* was found in the Malaysia waters.

The objectives of my participation in this training program are to gain help for accomplishing the projects on *Sargassum* and other brown algal systematic in Malaysia through molecular techniques; to standardize materials, methods and terms used in the molecular researches; and to support interdisciplinary research and several on-going researches benefit from the active collaboration between Algae Research Laboratory, IOES and other research institutions.

Total *Sargassum* samples that I have brought from Malaysia for the training program are 15 DNA samples and 7 silica gel dried samples. In this CAPaBLE training, it involved processes from DNA extraction (using QIAGEN plant extraction kit), step-down PCR amplification, gel electrophoresis, PEG PCR purification to cycle sequencing. From the DNA extraction results, a total of 17 samples were successfully amplified using PCR with the following markers: nad3-P1, mt16S-P1 and rps14-sm1. Primers used were designed for the particular species for better results and considered according to the suitability of the target gene. Successfully amplified PCR products were subjected to PEG purification and cycle sequencing. Four other primers were used for the cycle sequencing to produce a better result from the PCR amplified samples.

Results from the training program were shown in the following figure. *Sargassum binderi* was considered as an out-group for this analysis, a species belong to the subgenus *Sargassum* section *Acanthocarpicae*. The In-group were all belongs to the subgenus *Sargassum* section *Zygocarpicae*. The phylogenetic tree showed that only one specimen of Sargassum polycystum were clustered away from the other *Sargassum polycystum*, suggesting that there's a possibility of misidentification using the morphological taxonomy. From the result obtained, the molecular data was able to answer the confusion arise from the morphological taxonomy, which we can then check or confirm on the possibility of correct morphological identification of the species. Apart from that, more samples should be added for analysis to produce more confidents for the phylogenetic tree produced. New primers should also be designed according to the results obtained to produce sequences specific to the specimens in question.

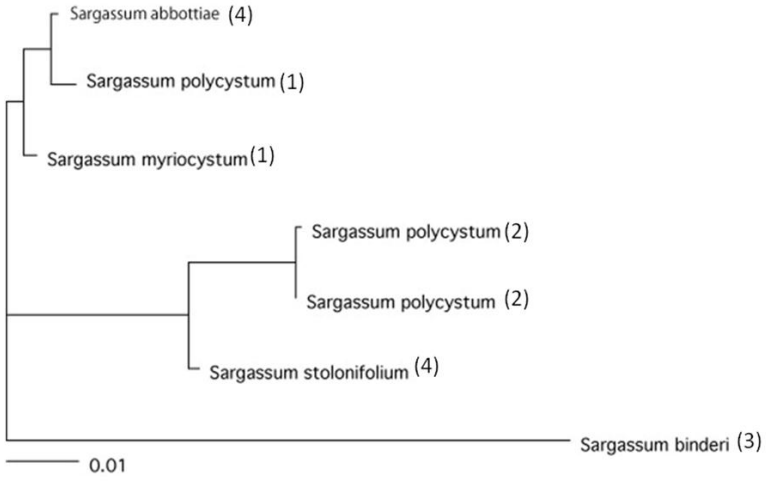


Figure: Phylogenetic tree estimated from the results obtained inferred using Maximum Likelihood criteria.

3. My opinion about the training in general

In my opinion, this training program provided a very good approach to train and educate new knowledge to the representative from each country. As the first attempt in promoting a network based on molecular analysis in compliment to the traditional taxonomy group, I think it has successfully carried out. During the training, we did exchange new information and collaboration among the participants. The knowledge we obtained is important for us to pass on to new students as well as build up the next generation of seaweed taxonomists for the university and country.

Although the training was short in terms of obtaining a good results to data analysis, the basic knowledge on the molecular were pass on due to one-on-one intensive daily training. Optimizations of each process of the molecular analysis normally take more time than the expected or the estimated period. For this, I would suggest that an extension of the period of training to a month for a more complete training on the data analysis. I believed that data analysis and tree interpretations are crucial for producing a better discussion on the results obtained.

- Anchana PRATHEP (Thailand)

CAPaBLE Training Report

My name is Anchana Prathep, I am an assistance professor at Prince of Songkla University, HatYai, Thailand. My background is more to do with seaweed ecology. I belong to Seaweed and Seagrass Research Unit, Department of Biology, Faculty of Science. The research is primarily concerned with the diversity, distribution, ecology and abundance of seaweed and seagrass at various sites in the South: Phuket, Trang, Satun, Chumporn, at Khanom-Mu Ko Thale Tai National Park, Nakhon Si Thammarat Province, and Songkhla. It includes seagrass monitoring under the ' Global SeagrassNet Monitoring' project, which we are now a hub in the South East Asian region. Studies have focus on the diversity and ecology of common species such as *Halimeda*, *Padina*, *Turbinaria* and *Dictyota.* Ecological theories have been investigated using seaweed and seagrass as "model organisms" in various habitats: intertidal, seagrass bed and shallow subtidal coral reefs. During the past 10 years, we have been looking at collecting the seaweed specimens and build up the reference collection as well as the database. We are now trying to move forward into the molecular study, which is a good tool to answer various research questions both taxonomy and ecology.

By attending the CAPaBLE workshop at Kobe University during 3-13 July 2010, this is a great opportunity for me to understand and have hands on experiences working on the molecular process which allow me to build up such research in Thailand. The training carefully walks us through every step that needed, allow us to understand and answer the research question that we are interested in. It was well set up for both the beginner and experience scientists in the field. The instructors have a great experience in the subject and clearly can walk us through the workshop and allow us to interact and ask any questions during the course. As zero knowledge in this field, I am very satisfied with what I have learnt during the workshop and I can ensure that this experience would be useful for our team in Thailand and it will be put into used in a very near future. I also really admired their dedication to the workshop and their trainee, they tried to checked cross what have went wrong with my technique/work/sample and spent time sit side by side and tried to find out the way to cross check my problems. This was great and this made my experiences during the course much happier and this gave me the sense of accomplishment that I can do it finally! I do have a tree- a Padina tree. I have also learnt that they are still a lot yet to learn and we can still develop several research questions using this molecular technique; and they are so much in details, time and energy and even money that needed to be able to accomplish such a great work that they have been doing. For the *Halimeda* systematic study, we have to really think carefully about it since they seem to be a bit more complicated than what we first thought, but at least it is a good start and I learnt that we really have to pay a good attention in every steps throughout the process from sample collection till.....

Although, the timing is rather short but it still allowed us to have a grasp at all aspects and

understand the molecular technique as a whole. The venue and facilities are great and the communication before coming to the workshop with the instructor is very useful and it helps the participants to prepare themselves or asks some questions if needed. The instructors are friendly and helpful; and also with the experience and authority, they could have certainly guide us for further work, which is rather important and useful.
I really do have a great time and look forward for further collaborations; and this program is very useful and would be benefit for the region.

- Soradkorn PHIMLA (Thailand)

CAPaBLE Training Final Reprot
Personal Data
Name: Soradakorn PHIMLA
Nationality: Thai
Present Position and Current Duies
Organization: Algal Bioresources Resaerch Center
Department: Department of Fishery Biology,
Faculty: Faculty of Fisheries, Kasetsart University, Bangkok, Thailand
Contact Address: Department of Fishery Biology, Faculty of Fisheries, Kasetsart University, Bangkok 10900, Thailand
Present Position: Researcher
Education: B.S. (Fisheries) Kasetsart University, Thailand 2003, M.S. (Fisheries Biology) Kasetsart University, Thailand 2009
Background and activity:
During I was an undergraduate student I was interested and worked in the field of biotechnology and ecology and taxonomy of the green seaweeds. My senior project was the determination of Preliminary Study of Polysaccharide Extracts and Morphology for Grouping Green Seaweed Genus *Caulerpa*.
After I graduated from the Faculty of Fisheries in 2003, I started my work at the Labolatory of Algae and Aquatic Plants of Department Fishery Biology, Faculty of Fisheries, Kasetsart University as a research assistant concerning with a cultivation of *Gracilaria* which focused on semi-enclosed system. In 2004, I started my work on Phenology and Biomass of *Caulerpa racemosa* var. *macrophysa* and Its Extracted Polysaccharide to complete master dissertation. During I am graduate student I was working as teaching assistant, with a subject of phycology at Department of Fishery Biology. I was appointed as research assistant concerning with survey of species diversity of *Gracilaria* in Thailand coastline for conservation of their stock and recovery on the natural coast. I am learning to get more experiences about molecular techniques from this work.
Current duties:
I working as researcher at Algal Bioresources Research Center (ABRC), I was appointed to undertake research project of “Carbon-Dioxide Reduction by Macro algae” at the ABRC. Moreover, I have been assist on morphological taxonomy and biodiversity of algal in Thailand under the Plant Genetic Conservation Project as the Royal Initiative of Her Royal Highness Princess Maha Chakri Sirindhorn at the Kasetsart University Museum of Fisheries (Natural and History). I have a plan to do research on the ecological taxonomy of *Laurencia* group. The taxonomy of these species is complicated due to morphological complex. I am focusing on the application of molecular genetics, together with morphological comparative to confirm and distinguish species of *Laurencia* complex.
Training Summary:
I have grateful opportunity to participate in “Capacity Building of Biodiversity Research in the Coastal Zones of the Asia Pacific Region: Phycology Taxonomy Analysis Training Using Genetic marker (APN CAPaBLE Program)” at Kobe University and Fukui Prefecture University, Japan from 4- 12 July 2010. The training program gives a lot of useful and completely respond for working as researcher at ABRC.

I hope to receive accurate analysis skill for seaweed identification using molecular marker when I come back to Thailand.

The CAPaBLE training was divided into three parts and was attended by approximately 20 persons. First was introduction meeting, Prof. Kawai and Dr. Furukawa Masakazu welcome remarked to participants. Then six of trainees including instructors and members of organizer introduced themselves and four special lectures on taxonomy of seaweed and plankton focused on using genetic marker.

In the practical training part, six trainees was divided in to three group depend on Division of seaweed. To analyses of phylogenetic relationship using molecgular data between six specimens of *Laurencia* Thailand and from Japan at Fukui Prefecture University under advice of Dr. Kamiya mitsunobu with Dr. Roike Iwan Monolalu from Indonesia who is working on Genus *Kappaphycus*. First of all, specimens were usually to check epiphyte that contaminant on DNA extraction. There were several methods and techniques for working on molecular genetics and phylogenetics in seaweed, especially in *Laurencia* group. The pressed specimens of *Laurencia majuscula*, *L. snackeyi* and *L. mariennensis* from Thailand and *L. marjuscula.*, *L. snackeyi* and *Chondrophycus cartilaginous* from Japan are used in molecular analysis. Total cellular DNA was extracted using GenEluteTM Plant Genomic DNA Miniprep Kit (Sigma-Aldrich Japan K.K., Tokyo, Japan) and Modified Chelex method. The polymerase reaction and sequencing primer used for all specimens were F-*rbc*L start, F598, F1165 and R-*rbc*S start R-269, R- 680, R-1247. Protocols used for PCR and Sequence- amplification were applied by using KOD FX and BigDye® Terminator v3.1 Cycle Sequencing Kit. The sequence obtained were aligned for phylogenetic analyses using the CLUSTAL X algorithm with MEGA vers. 5 Beta 4 and refined by eye. The Phylogenetic trees were inferred using neighbor-joining (NJ) method and boostrap analyses were performed to assess the robustness of clades (1,000 replicates.) The result of molecular analyses shown only three sequences data of specimens from Japan were obtained. Probably the primer and conditions of amplification were not suitable with Thai specimens. Finally, the presentation of practical training from six trainees were produced in last day of training program at Kobe University. The result of phylogenetics analyses were discussed by instructors and invited commentator. Even if I was unsuccessful to analyses molecular of *Laurencia* spp. from Thailand, but I have learned several method, received nice relationship and great experience.

- Minhthanh Thi NGUYEN (Vietnam)

CAPaBLE TRAINING REPORT

I. Introduction and background

1. Name: NGUYEN THI MINH THANH
2. Position: Researcher
3. Organization: Department of Algal Biotechnology, Institute of Biotechnology, Vietnam Academy of Science and Technology, Hanoi, VIETNAM
4. Office Address and Contact Information:
   Address: Institute of Biotechnology, Vietnam Academy of Science and Technology, 18 Hoang Quoc Viet, Cau Giay, Hanoi, Viet Nam
5. Current main researchs:
   * Culture marine microalgae in laboratory scale.
   * Exploitation of natural bioactive compounds from seaweed of Vietnam.
   * Study on conversion of marine algal biomass to biodiesel.
   * Study on the taxonomy analysis based on the morphological characteristics and using genetic markers.

6. Job Record (After graduation)

Organization	City/ Country	Period		Position or Title	Brief Job Description
		From Month /Year	To Month /Year		
Algal Biotechnology Department, Institute Biotechnology (IBT), Vietnam Academy Science and Technology	Ha Noi, Viet Nam	11/Nov., 2006	now	Researcher	Work on microalgae and macroalgae (culture, extraction active compounds, conversion marine algal biomass to biodiesel, the taxonomy analysis of seaweed based on the morphological characteristics and using genetic markers. …)
Hue University	Hue city, Viet Nam	03/2006	10/2006	Researcher	Plant tissue culture

7. Educational Record

Institution	City/ Country	Period		Degree obtained	Major
		From Month /Year	To Month /Year		
University of Malaya, Malaysia	Malaysia	8 April	15 April	Participant of the 2nd SEASTax Workshop	Taxonomy analysis of seaweed
Institute of Ecology and Biological Resources, Vietnam Academy Science and Technology	Ha Noi, Viet Nam	2008	2010	Master	Biochemistry
Hue University of Science	Hue city, Viet Nam	2002	2006	Bachelor	Biochemistry

II. Training Summary

Molecular Taxonomy of Marine Macroalgae (Hokkaido University, July 5 – 9, 2010).

The training contents:

Phylogenetic analysis using *Ulva* species for the ITS region:

(1) Samples for DNA extraction – (2) DNA extraction – (3) DNA purification with Standard Super-Cel – (4) PCR – (5) Agarose gel electrophoresis – (6) PEG precipitation (Purification of PCR products) – (7) Cycle sequencing – (8) Purifying cycle sequencing products – (9) Electrophoresis on ABI 3130 genetic

analyzer – (10) Aligning DNA sequences – (11) Phylogenetic analyses using PAUP.

III. Opinion about the training in general
Identification methodology using genetic markers is recognized these days to reinforce the shortcomings of traditional approaches. So, this training program aims to facilitate the basic skills about phylogenetic taxonomy using genetic marker and taxonomical information exchange among young researchers from South East Asian countries. As for me, this training program is very usefull for my researchs because in Vietnam there are not enough experts to help our laboratory in phylogenetic taxonomy. I have learned many value experiences about genetic marker and application in phylogenetic taxonomy. In addition, I met many experts and young researchers from other countries who are all concern about phylogenetic taxonomy. After this training program. I will try my best to apply these lessons in my researching and for further studies in my laboratory and in my country.

2011
- Phala CHEA (Cambodia)

CAPaBLE Training Report
1. Introduction and background
Personal Data
Name: CHEA PHALA
Nationality: Cambodian
Office address: Marine Aquaculture Research and Development Center, Fisheries Administration, Preah Sihanouk City, Cambodia
Education Background
1996-2001: Bachelor of Aquaculture, Research Institute for Aquaculture No1, Ha Noi, Vietnam.
2001-2003: Master of Agriculture, Research Institute for Aquaculture No1, Ha Noi Agricultural University No1, Vietnam.
2003-2007: Ph.D in Biology, Research Institute for Marine Fisheries, Hai Phong, Vietnam.
Working background
2003-2007: Working in Division of Marine Fisheries Resources of Research Institute for Marine Fisheries, Vietnam.
2008-2009: Lecturer of Meanchey University, Subject is Marine biology, in Cambodia.
2009-2010: Senior officer of Freshwater Aquaculture Research and Development Center, Fisheries Administration, Cambodia and National Component Director of Aquaculture Indigenous of Mekong Species Cambodia Sub-component of Mekong River Commission.
2010 up to now: Acting Director of Genetic and fish disease Division of Marine Aquaculture Research and Development Center, Fisheries Administration, Cambodia.
2. Training summary
The training program held from December 03 to December 13, 2011 was attended by six trainees from five countries. Trainees were divided into three groups and three different universities for their individual technical training namely Kobe University, Hokkaido University, and Fukui Prefecture University. The time of training, total is 10 days, but 2 days travel and 2 days for welcome remark and sum up and closing so remain 6 days for practice. Fukui Prefecture University has good infrastructure and facilities for training and research. The training practice started from sample collection on field to analysis took time 6 days. During the practice we tried to use two different methods for DNA extraction. We spent all time to practice and learn how to extract DNA, how to amplify PCR, how to check PCR products, how to purify PCR product, cycle sequencing, sequencing and analyze the data. Each process is quite complicate but easy to follow. We could not remember all the process but we noticed so we can do again by easier. Finally, we got the good result and discussion. This training provides not only the technique but also any comments from instructors

and professors to implement in own country. So this training program provided a very good approach to training and providing the representatives from each country with new knowledge. 10 day is not enough to complete all process but could help participants how to conduct research and update their knowledge.

3. My opinion about the training

Taxonomy of marine macroalgae using molecular markers is a new technology and very convenience for scientist to compare among phylogenetic distant organisms, various genetic information, stable against environmental and developmental variation, easy to define theoretical models and apply statistical analyses, and possible to estimate divergence time. In my opinion, this training was very important for participants that provided not only new knowledge but also gave opportunity to create strong networking amongst participants and countries. As the first attempt in promoting a network based on molecular analysis in a way that is complementary to the traditional taxonomy group, the training was carried out very successfully. The knowledge we obtained is important for us, especially for country poor information and limit in biodiversity research like Cambodia.

- Tsz Yan NG (China)

CAPaBLE Training Program Report

Trainee: NG Tsz Yan

Training period: 4th Dec – 12th Dec 2011

I am a newly admitted postgraduate student from the Chinese University of Hong Kong (CUHK), studying the PhD program of Biology, School of Life Sciences and carrying out my research in the Marine Science Laboratory of CUHK. Previously, as my final year project of undergraduate study, I worked on the classification of dinoflagellate of the genus *Symbiodinium* which forms symbiosis with scleractinian corals in Hong Kong, using molecular approach. There are different clades of *Symbiodinium* which can be distinguished only by molecular methods. Hosting different clades of *Symbiodinium* probably can directly affect the physiological performance, such as thermal resistance, of the corals. Therefore, knowing which clades of *Symbiodinium* that different scleractinian corals are hosting would be important for the conservation of the coral reefs. As part of my postgraduate study, I propose to expand the work of classification of *Symbiodinium* in Hong Kong into subclade level. Hopefully it will provide a clearer picture of the *Symbiodinium* diversity and taxonomy in Hong Kong, which can then be used as baseline information for further investigation on dinoflagellate as well as coral conservation in Hong Kong.

During the CAPaBLE training period, I was assigned to the Hokkaido University. I work under the supervision of Dr. Horiguchi and Dr. Kogame of the Phycological Laboratory, Faculty of Science, to learn the knowledge and techniques on molecular taxonomy of marine macroalgae. Below is the schedule of the training:

Dec 4 Travelling from Kobe to Sapporo
Dec 5 DNA extraction, DNA purification
Dec 6 PCR, Electrophoresis
Dec 7 PEG precipitation, Cycle sequencing
Dec 8 Purification, Sequencing
Dec 9 Phylogenetic analysis
Dec 10 Traveling from Sapporo to Kobe

Here I will make a summary of the training contents. For DNA extraction from algae, I have tried a method modified by the laboratory, using samples of two different algae species. And then I purified the extracted DNA with standard super-cel and NEW Wash solution in order to obtain high quality DNA for Polymerase Chain Reaction (PCR). In PCR, I learned how to prepare a reaction mixture with proper proportion and tried different sets of primers in order to get the best results. To check for the PCR products, I prepared an agarose gel and preformed electrophoresis to look for clear bands which indicate successful PCR. Purification of PCR products was then done using PEF precipitation.

Then the PCR products can undergo cycle sequencing to insert fluorescent probes in different sequence length. After that, precipitation using ethanol, EDTA and sodium acetate was preformed to purify the products of cycle sequencing. The products were then put into a genetic analyzer for electrophoresis and then generating the electrophograms. The DNA sequences of the samples were then manually aligned with DNA sequences of other known species, which could be downloaded from DNA sequences database such as GenBank and DDBJ. This step was done by using PAUP*, a program for phylogenetic analysis. Finally, a phylogenetic tree was constructed with PAUP*, using maximum parsimony. Bootstrap analysis was also done to check for the validity of the branches of the tree. With the phylogenetic tree, the taxonomic relationship of the sample algae with other algae species can be evaluated.

I am very satisfied with the whole training program. With some basic knowledge of molecular techniques, I find the training contents very useful in improving my techniques. The training as well as the lectures given on the first day also provided me more details and new insights on the application of molecular methods on taxonomy of marine macroalgae, as well as other marine organisms. Among the training contents, cycle sequencing and sequencing with a genetic analyzer are fully new to me. After getting familiar with the working principle through the training, undoubtedly I will be able to get better results when using the same techniques again. Moreover, I think the training is very practical. I manage to gain hand on experiences which are really impressive during the training. Here I have to say thank you again to Dr. Horiguchi and Dr. Kogame. With their instruction step by step, I understand each procedure very clearly and I am confident to be capable to apply those technique again on taxonomy analysis.

Furthermore, I believe the program has successfully helped to build up a network among researchers for information exchange. Trainees meet and get on well with each other throughout the training program. So it should be possible for us to share taxonomical information in the near future.

It is my honor to be selected as one of the trainees of the CAPaBLE training program. After the training and going back to my country, I will share the experience and techniques I gained from the program with others researchers in Hong Kong. Hopefully it will help and lead to more people to work on molecular phylogenetic as well as evaluation of the biodiversity of macroalgae in this region.

- Phaik Eem LIM (Malaysia)

Report for CAPaBLE Training Program by Phaik Eem LIM

Introduction and Background

I am currently working as a Senior Lecturer at the Institute of Biological Sciences, University of Malaya. I have involved in the study of the biodiversity, taxonomy and molecular phylogenetic of marine seaweeds for more than 10 years. I am currently leading the molecular phylogenetics group of the Algae Research Laboratory, University of Malaya. At this stage we have several on-going projects on the taxonomy of algae using molecular markers such as on *Gracilaria*, crustose brown seaweeds, *Gelidiales* and *Chlorella*. The Algae Research Laboratory is also researching on the molecular phyolgenetics of cultivated *Kappaphycus* species and genetic diversity on cultivated *Kappaphycus alvarezii* in South East Asian countries. Resulting from the SEASTax (South East Asian Seaweeds Taxonomy) II, Dr. Dang Diem Hong from Vietnam and Dr. Anicia Hurtado from Philippines and later on through MOU (memorandum of understanding) between Institute of Ocean and Earth Sciences (IOES) and Mataram University, Prof Sunarpi from Indonesia has agreed to work on this collaborative research. To date, our research group has managed to use the published molecular markers, plastid encoded: *Rubisco* spacer and mitrochondrial encoded marker: cox2-3 spacer in elucidating the taxonomic position of Malaysian specimens. The mentioned molecular markers are suitable for examining the taxonomic position of *Kappaphycus* at species level but not variable enough for genetic diversity. The next phase of the project, we will focus on the genetic diversity of *Kappaphycus alvarezii* from South East Asian countries.

I am also being appointed as the coordinator for the Molecular Biology Section of IOES, University of

Malaya. I am also assisting the molecular phylogenetics genetic study of the marine invertebrates.

Training Summary

The opportunity to attend the CAPaBLE training program is an immerse opportunity for me to learn new molecular techniques for the taxonomy of seaweeds. I was assigned to be based at the Kobe University to learn the molecular techniques from Prof. Hiroshi Kawai, Assistant Prof. Dr. Takeaki Hanyuda and Dr. Akira Kurihara.

In this training, two objectives were planned:

(i) An attempt to search for suitable molecular marker for the genetic diversity on *Kappaphycus alvarezii*

(ii) Learning new technique on Single Strand Conformation Polymorphism (SSCP) and its application in species identification for *Kappaphycus* and *Eucheuma*.

For the first objective, I was given the opportunity to try on the primer sets that was designed from the region in between cox3 to cob of mitochondrial region of *Chondrus crispus*. One of the primer sets managed to amplify only on some of the *Kappaphycus alvarezii* samples. The PCR products on the successfully amplified samples were subjected for sequencing and based on the generated sequences, another new primer sets was designed. This new primer set managed to amplify all *Kappaphycus alvarezii* samples. The partial sequences of the amplified region, which was from tRNA Leu to cob of mitochondrial region showed six haplotypes out of the six samples that were examined. The base pair differences ranged from 1 to 10 bp.

For the second part of the training, I was taught on how to carry out the SSCP experiment. For his purpose, we have selected partial sequences of cox1 region. Two primers were designed based on cox1 sequences of *Kappaphycus* and *Eucheuma* which were carried out in Malaysia. With the new primers and the common bar coding marker for algae GazF1, we managed to amplify the DNA samples of *Kappaphycus alvarezii*, *K. striatum*, *Kappaphycus* sp., *Eucheuma denticulatum*. Two experiments were carried out for SSCP. SSCP of partial cox1 generated unique DNA fingerprints patterns for each species and this can differentiate the four respective species: *Kappaphycus alvarezii*, *K. striatum*, *Kappaphycus* sp., and *Eucheuma denticulatum*.

Opinion on the Training

First of all, I would like to take the opportunity to thank the organiser of this training program: International EMECS Center, Kobe University, Hokkaido University and Fukui Prefecture University and the financial support from Asia Pacific Network (APN) in organising this training program for the young researchers from South East Asia in equipping us with molecular techniques for taxonomy study.

This training has allowed me to learn a new molecular DNA fingerprinting method - single strand conformation polymorphism (SCCP) which will be very useful for my future research. In addition to this, it also gives us the opportunity to foster the networking between South East Asia participants and Japanese researchers.

Even though the hand on training was conducted in a very short period of time (5 days) but early and active communication via email with my hosts in discussing the training objectives has helped me to achieve much in this training course. My host and I have started the communication one month in advance. A special thank is due to my hosts at Kobe University for their dedication and initiative in helping me to achieve the planned two objectives.

A warm hospitality from the staff of the International EMECS Center right now the start in arranging the visa application, flight schedule, transport arrangement, accommodation and etc has make our stay at Japan comfortable warm and pleasant.

In summary, I have learned a lot and gained valuable molecular technique for taxonomy purposes through this CAPaBLE training program.

- Sze-wan POONG (Malaysia)

REPORT FOR CAPaBLE TRAINING 2011 (3-13 DECEMBER 2011)
BY: POONG SZE WAN (INSTITUTE OF BIOLOGICAL SCIENCES, UNIVERSITY OF MALAYA, MALAYSIA)

First of all, I would like extend my gratitude and appreciation to the Asia Pacific Network for Global Research (APN) for sponsoring this training programme and heartiest congratulations to the International EMECS Center for a job well done in organizing this programme. I am as of the present, a third-year PhD student under the supervision of Dr. Lim Phaik Eem and Prof. Dr. Phang Siew Moi, based in the Institute of Ocean and Earth Sciences in University of Malaya, Kuala Lumpur, Malaysia. I started my Bachelor of Sciences study majoring in Biotechnology back in 2006 in which my final year project on the phylogenetics of *Lobophora variegata*, a brown macroalgae, was also conducted under the supervision of Dr. Lim Phaik Eem and Prof. Dr. Phang Siew Moi.

My current research interest is on the taxonomy and molecular phylogeny of crustose or encrusting brown algae in Malaysia. These algae have been poorly studied, more so in the molecular aspect despite some of them being the dominant flora in some habitats, due to their unattractive appearances and no obvious economic value. Nevertheless I believe a study dedicated to these algae marks a better understanding of the biodiversity of the macroalgae in our region. Molecular analyses form an essential aspect of my study and this is due to the relative simplicity of my specimens in terms of their morphological characters which are rather lacking. This makes identification up to even genus level an uphill task. Reproductive structures which are important in the identification of encrusting brown algae are unfortunately seasonal and elusive in the field, thus hampering the identification process. This is where molecular analyses come into the picture. During the course of my research, I tested a number of genetic markers in terms of their resolution from the ordinal to the species level and ease of amplification, to name a few.

For the purpose of this training programme, I brought along some of my own dried specimens to be worked on. During the practical training in Hokkaido University, under the supervision of Professor Takeo Horiguchi and Professor Kazuhiro Kogame, I performed DNA extraction on two of my samples of *Mesospora schmidtii*. Back in Malaysia, we used commercial DNA extraction kits which are rather costly and this time round, I had the opportunity to perform conventional DNA extraction using a low cost modified method used in Professor Kogame's laboratory. This method appeared to work quite well for my samples as evidenced by the success in PCR amplification. Besides that, I was exposed to DNA purification using the Standard Super-Cel suspension. As suggested by Professor Kogame, I tested two plastid-encoded genes, the psaA gene which encodes the PS I P700 chlorophyll A apoprotein A1 and the psbA gene which encodes the PS II thylakoid protein D1. The procedures of PEG precipitation, cycle sequencing and electrophoresis on a genetic analyser are new experiences for me as we use purification kits in my own laboratory and sequencing services was carried out by a company.

My overall opinion about this training programme is that it represents a good platform for each participant to gain new knowledge and skills and in my case, put into perspective what we have learnt in theory. I have to admit that the practical training period of five days was rather short and a longer period of time may prove to be a more fruitful experience. Nonetheless, the programme serves as a good opportunity for young researchers like us to build up our network among various countries. I believe that each and every one of us would be most happy and delighted to share the knowledge that we have picked up from the training programme with our fellow colleagues in our laboratories or research centres. Although most of us are currently working on macro and microalgae, the techniques we learnt are also applicable for use in other marine organisms. Thank you once again to all parties involved in making CAPaBLE 2011 a success! Arigato gozaimasu!

- Narongrit MUANGMAI (Thailand)

Report for CAPaBLE Training Program
Phycology Taxonomy Analysis Training Using Genetic Marker
December 3-13, 2011

My name is Narongrit MUANGMAI from Thailand. I am currently a research assistant at Department of Fishery Biology, Faculty of Fisheries, Kasetsart Univerity (KU), Thailand. I earned B.Sc. (Fisheries), specializing in fishery biology field, from the Faculty of Fisheries, KU in 2006, and then received the support from the Japanese Government Scholarship to continue my study in Japan. I obtained my M.Sc. (Agriculture), emphasizing in animal and marine bioresources science field, from Kyushu University, Japan in 2010 under the supervision of Prof. Shigeo KAWAGUCHI. My research interests are in the area of taxonomy and evolution of marine red algae. My works in KU are evenly divided between taxonomic investigation of coralline red algae by using microtome techniques and filed survey of marine plant biodiversity.

During the CAPaBLE training program for macroalgal taxonomy by using molecular markers in 2011, I had an opportunity to learn and work under the guidance of Prof. Hiroshi KAWAI, Assist. Prof. Takeaki HANYUDA and Dr. Akira KURIHARA at Kobe University research Center for Inland Seas. In the center, I have learned many of the new techniques for applying molecular techniques to resolve the algal taxonomic problem and detect the marine algal invasion. Regarding to my work for this training course, I brought Thai samples of small-flattened species of marine red algal, *Gracilaria* (*Gracilariaceae*, *Rhodophyta*), which was collected from Andaman Sea, south Thailand, for verifying its identity by using molecular data, together with the detailed comparative morphology.

Molecular works were spilt into laboratory exercise and computational analysis. With respect to laboratory work, DNA of Thai *Gracilaria* specimens was extracted using the modified method developed by Dr. Hanyuda, and then produced a million of copy of specific region of *rbc*L gene (plastid DNA) with PCR techniques. Thereafter, agarose gel electrophoresis was done to check the results of PCR amplification. PCR products were purified by PEG method, and finally cycle sequencings and DNA sequencing were done successfully. After getting the 1112 bp sequence of *rbc*L gene, multiple sequence alignment was performed using eBioX and MacClade software and then imported as FASTA format file. Substitution model selection for the dataset was carried out with Kakusan4. Maximum likelihood and Bayesian method were selected as a method for reconstructing phylogenetic tree, and implemented by Phylogear and MrBayes software respectively. Bootstrap value and Bayesian probability were also calculated for inspecting the reliability of each clade of phylogenetic tree.

Our resulting phylogentic tree shows that Thai *Gracilaria* specimens are located in a separated clade from other known flattened *Gracilaria* species of Asia Pacific region. Thai *Gracilaria* species seems to be split early in evolutionary lineage of Asia Pacific flattened species of *Gracilaria*. Additionally, the reproductive organs of Thai species are relatively different from other flat species of *Gracilaria*. The spermatangial conceptacle of Thai *Gracilaria* specimen shows a verrucosa-type configuration; while other Asia Pacific flattened species of *Gracilaria* are textorii-type. As a result, a small-flattened species of Thai *Gracilaria* should be proposed as a distinct in genus *Gracilaria.*

From the first day till the last day of CAPaBLE training course here in Kobe University, I am very happy to learn from all adroit Japanese phycologists. This intensive training course enables me to work close to specialist in the field of phycology and molecular biology, and we have shared a lot of experiences not only in academic aspects, but also in general aspects. For the academic issues, I can learn how to make an effective phylogenetic tree using various kinds of software, such as Kakusan4, Phylogear and RAxML, which I have never worked on it before. I also had a lively discussion with my professor and colleagues regarding to how we can build up a strong network on algal researches in Southeast Asian countries.

Furthermore, this workshop gives me a window of opportunity to create a dialogue with other participants. We are from different places, different community and different culture, so when we

come together we have a lot of things to talk and share. We even talked over about the phycological research network in our region for the near future. Hopefully, we can do all as we discussion and make our network stronger.
Although the time for training is quite limited, but I am very confident that I get all that I expect to learn before coming here. No matter how long this training course I strongly believe that all instructors try their utmost best to teach and train all of us. However, for the participants who are new in the field of phycology and genetics, more time in training might benefit them for gaining more experiences in research.
Given a chance to join this training activity by APN and EMECS, I earn more experiences in research and can make the algal research network with Japanese and Southeast Asian phycologists. Million Thanks for a good time here in Japan. "Arigato Gozaimasu"
Narongrit MUANGMAI, December 13, 2011

- Ngocmai Thi DINH (Vietnam)

CAPaBLE Training Report
1. Introduction and background
Name of the trainee: DINH THI NGOC MAI
Nationality: Vietnam
Position: Researcher
Department: Algal Biotechnology Department, Institute of Biotechnology, Vietnam Academy of Science and Technology
Office address: Institute of Biotechnology, Vietnam Academy of Science and Technology, 18 Hoang Quoc Viet Street, Cau Giay District, Hanoi, Vietnam
Outline of duties: Culture marine microalgae in laboratory scale, exploitation of natural bioactive compounds from seaweed of Vietnam, study on conversion of marine algal biomass to biodiesel, study on the taxonomy analysis based on the morphological characteristics and using genetic markers.
Education report
2010 – 2012: Master, Institute of Ecology and Biological Resources, Vietnam Academy Science and Technology
2004 – 2008: Bachelor, Hanoi University of Science
Job report
11/2008 – now: Researcher, Department of Algal Biotechnology, Institute of Biotechnology (IBT), Vietnam Academy of Science and Technology
2. Training Summary
4 Dec (Sun): Introduction Meeting (Venue: Kobe University)
5 –9 Dec: Practical Training at Faculty of Marine Bioscience, Fukui Prefectural University. Supervisor is Prof. Mitsunobu KAMIYA
11 Dec (Sun): Summary Meeting
Report on the achievement and perpective of future research program
Summary of my report
Ulva is reportedly good source of protein and vitamins and is usually used in Japan and other East Asian countries for human consumption. Characterization based on morphometric characters created confusion for exact identification of the same. Hence, the need arises to identify these species precisely based on molecular tools. I applied molecular marker techniques in taxonomic identification of a *Ulva* species collected from Obama Bay. Some techniques I used in this study are DNA extraction (using GenElute Plant Genomic DNA Miniprep Kit and modified Chelex extraction of DNA), PCR, check of PCR products, PCR products, cycle sequencing, sequencing, confirmation and edit od sequencing, phylogenetic analysis. The obtained results suggested that *Ulva* species collected from Obama Bay identical to sequences belonging to *Ulva linza* or *Ulva prolifera*.

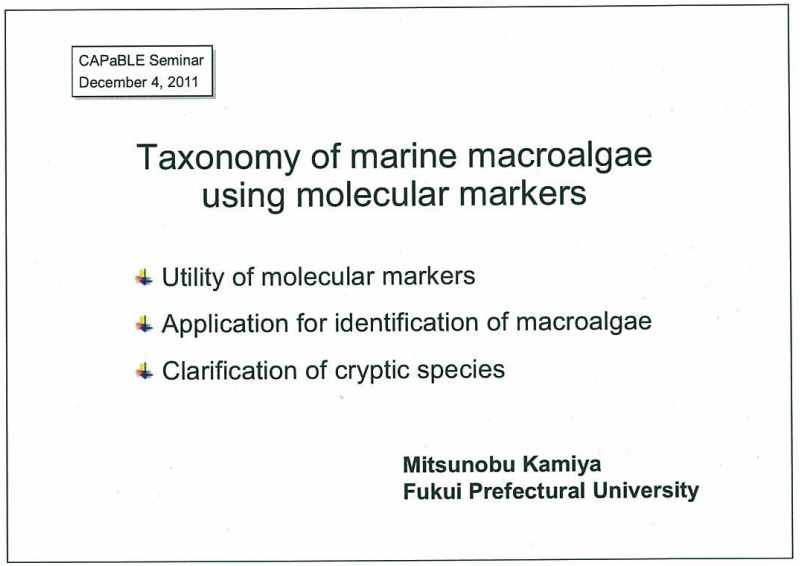
3. The opinion about the training in general

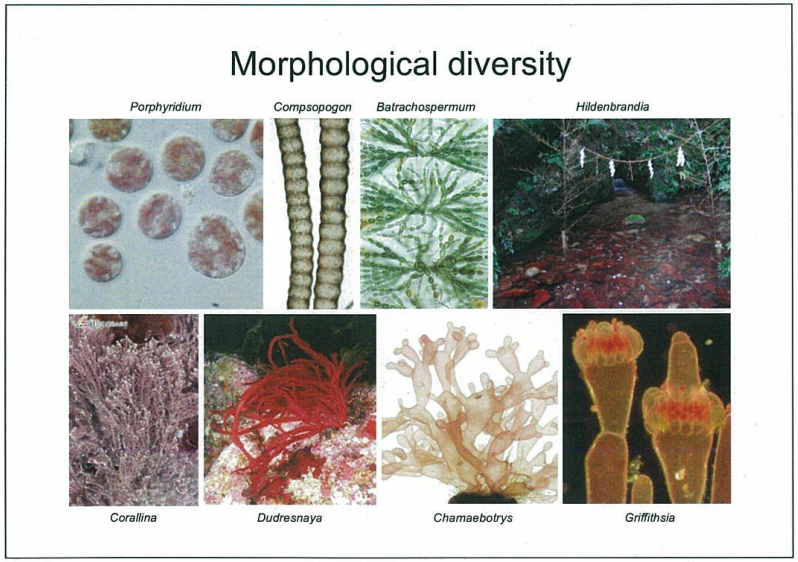
I think the training was carried out very successfully. Training program aimed to facilitate taxonomical information exchange among researches and to conduct rapid assessments with a newly developed skill of taxonomy identification with genetic marker. After the training program, I will share the skills and information with other researchers and apply the basic identification skill of genetic marker in the analysis of micro and macroalgae and other organism. I would like to suggest an extension of the period of training to one month, so as to achieve more complete training in data analysis.

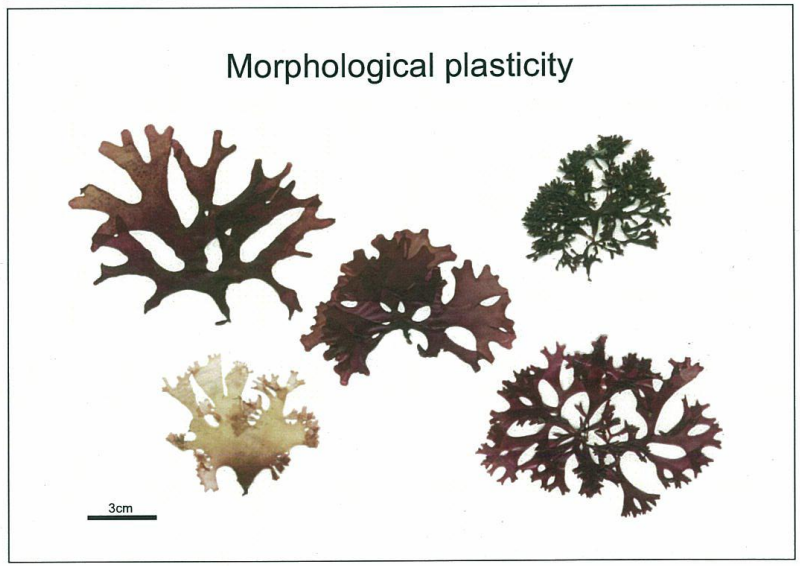
Appendix 4 Glossary of Terms

ABI	Applied Biosystems
BLAST	basic local alignment search tool
CTAB	cetyl trimethyl ammonium bromide
DDBJ	DNA data bank of Japan
DGGE	denaturing gradient gel electrophoresis
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleic acid triphosphate
EDTA	ethylene diamine tetraacetic acid
FFPE	formalin-fixed, paraffin-embedded
ITS	internal transcribed spacers
Leu	leucine
LSU	large subunit
MEGA	molecular evolutionary genetics analysis
NCBI	National Center for Biotechnology Information
PAUP	phylogenetic analysis using parsimony
PCR	polymerase chain reaction
PEG	polyethylene glycol
psu	practical salinity unit
*rbc*L	*rbc*: ribulose-1.5-bisphospate carboxylase/oxgenase (Rubisco) L: large
*r*DNA	ribosome DNA
RFLP	restriction fragment length polymorphism
rpm	revolutions per minute
SEASTax	Southeast Asian Seaweed Taxonomy
SSCP	single strand conformation polymorphism
SSU	small subunit
Taq	*Thermus aquaticus*
UV	ultraviolet


Fukui Prefectural University (Obama campus)
Seafood Market Research
Marine Bioengineering and Aquaculture
Ecosystem Enginnering
embryology of flatfish
immunology of blowfish
extraction of useful compounds
Coastal Oceanography
Food Technology
Environmental Microbiology


CAPaBLE Seminar
December 4, 2011
Taxonomy of marine macroalgae using molecular markers
Utility of molecular markers
Application for identification of macroalgae
Clarification of cryptic species
Mitsunobu Kamiya
Fukui Prefectural University


Morphological diversity
Porphyridium
Compsopogon
Batrachospermum
Hildenbrandia
Corallina
Dudresnaya
Chamaebotrys
Griffithsia


Morphological plasticity
3cm

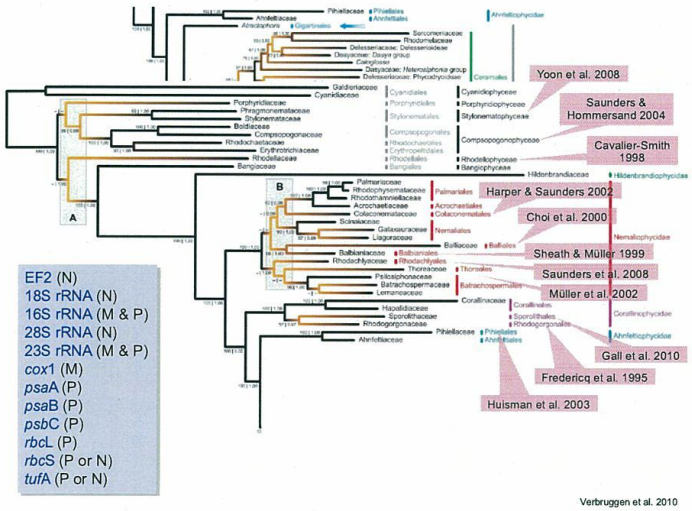
Advantages of molecular markers

- ✓ Able to compare among phylogenetically distant organisms
- ✓ Available of various genetic information
- ✓ Stable against environmental and developmental variations
- ✓ Easy to define theoretical models and to apply statistical analyses
- ✓ Possible to estimate divergence time

Evolutionary rate

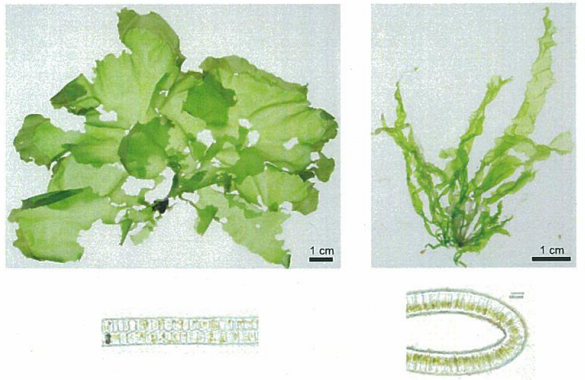
Comparison of partial leptin between mouse and human

Protein	Substitution rate per amino acid ($\times 10^{-9}$)
fibrinopeptide	8.30
ribonuclease	2.10
lysozyme	2.00
hemoglobin α	1.20
myoglobin	0.89
insulin	0.44
cytochrome c	0.30
histon H4	0.01



Ulva vs *Enteromorpha*

Ulva pertusa | *Enteromorpha compressa*



Enteromorpha is a synonym of *Ulva*

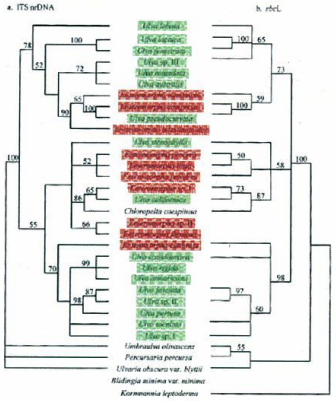


Fig. 1. Comparison of strict consensus trees derived from (a) nuclear ribosomal ITS sequence data and (b) the chloroplast-encoded *rbc*L gene. Bootstrap percentages (1000 replicates samples) are shown above branches. Nodes with bootstrap values of less than 50% are not labelled.

Hayden et al. (2003)

Ulva prolifera

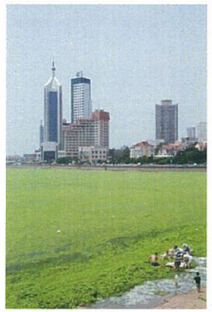
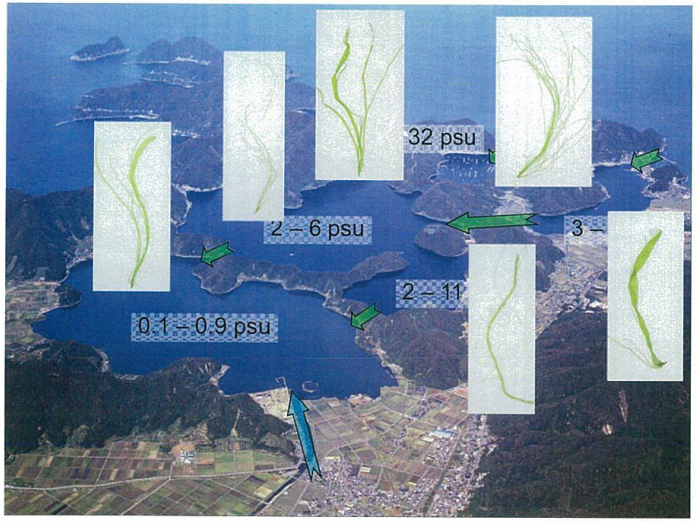
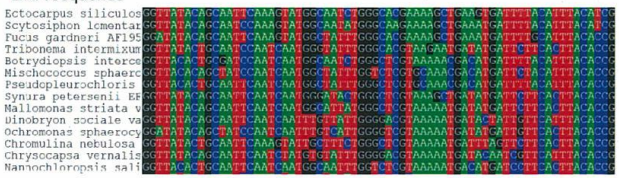


Photo: Hiraoka

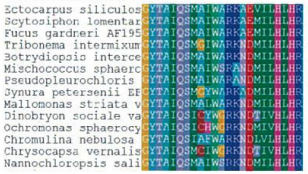


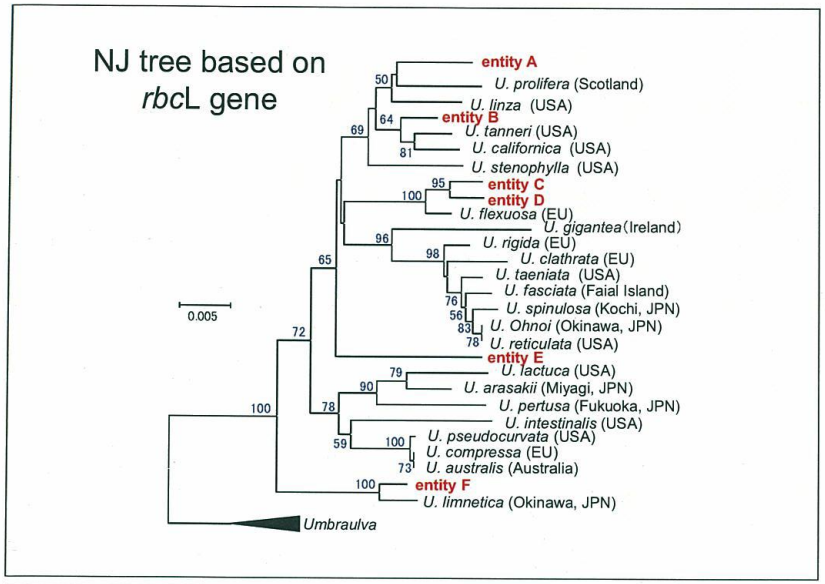
ribulose-1,5-bisphosphate carboxylase/oxygenase, large subunit (*rbc*L)

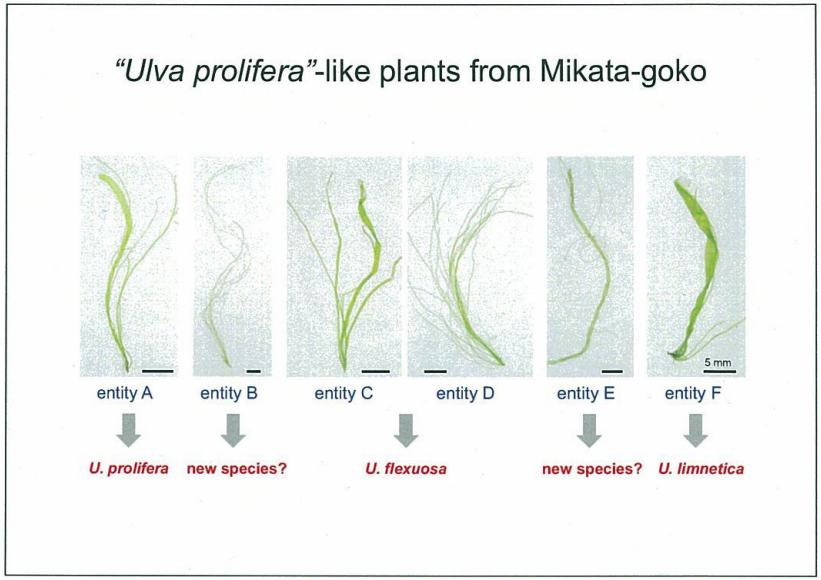
DNA sequence

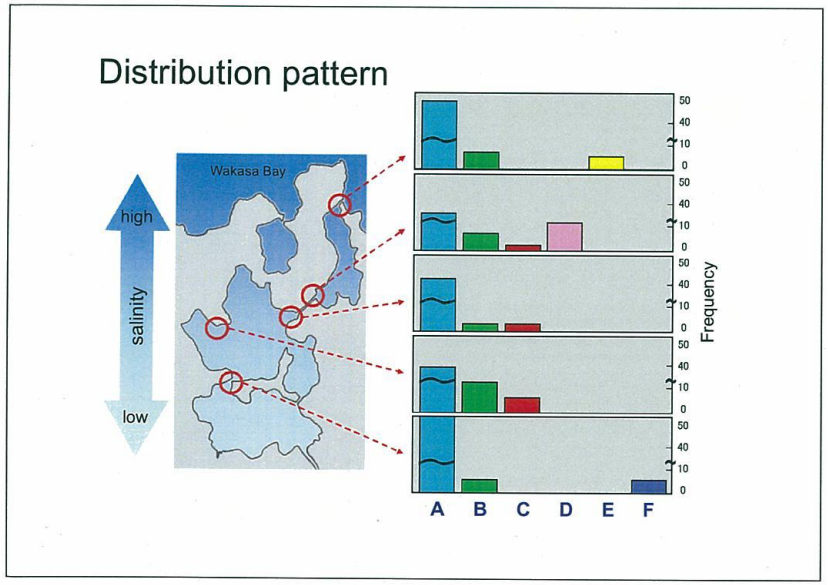


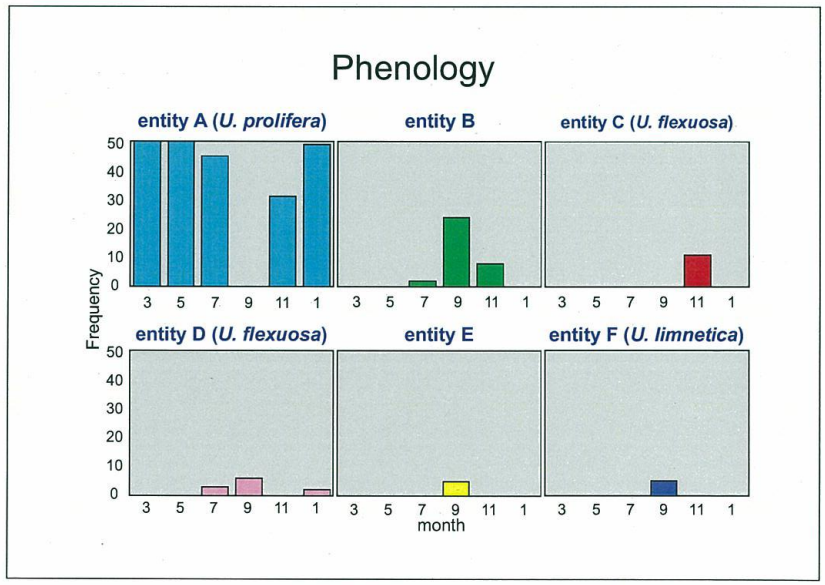
amino acid sequence



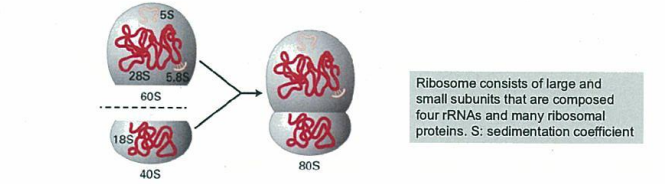

NJ tree based on
rbcL gene
entity A
U. prolifera (Scotland)
U. linza (USA)
entity B
U. tanneri (USA)
U. californica (USA)
U. stenophylla (USA)
entity C
entity D
U. flexuosa (EU)
U. gigantea (Ireland)
U. rigida (EU)
U. clathrata (EU)
U. taeniata (USA)
U. fasciata (Faial Island)
U. spinulosa (Kochi, JPN)
U. Ohnoi (Okinawa, JPN)
U. reticulata (USA)
entity E
U. lactuca (USA)
U. arasakii (Miyagi, JPN)
U. pertusa (Fukuoka, JPN)
U. intestinalis (USA)
U. pseudocurvata (USA)
U. compressa (EU)
U. australis (Australia)
entity F
U. limnetica (Okinawa, JPN)
Umbraulva
0.005


"Ulva prolifera"-like plants from Mikata-goko
5 mm
entity A
entity B
entity C
entity D
entity E
entity F
U. prolifera
new species?
U. flexuosa
new species?
U. limnetica

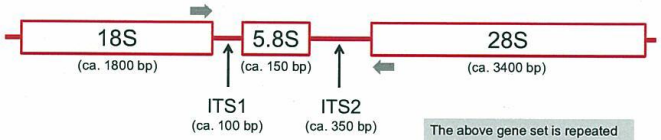

Distribution pattern
Wakasa Bay
high
salinity
low
Frequency
A B C D E F


Phenology
entity A (U. prolifera)
entity B
entity C (U. flexuosa)
entity D (U. flexuosa)
entity E
entity F (U. limnetica)
Frequency
month

Internal Transcribed Spacer (ITS)

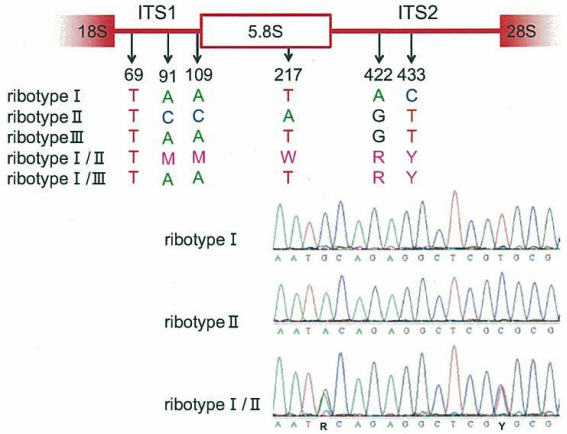


Ribosome consists of large and small subunits that are composed four rRNAs and many ribosomal proteins. S: sedimentation coefficient

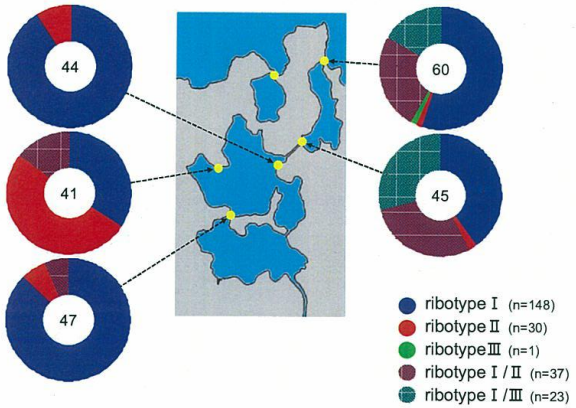


The above gene set is repeated tandem on a chromosome. ITS regions are removed after transcription.

Genetic variation within *U. prolifera*

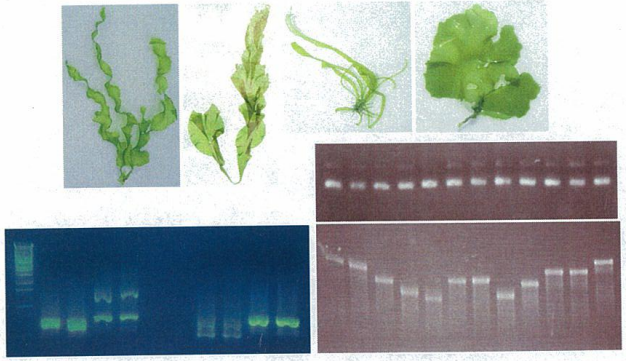


Distribution of *U. prolifera* ribotypes



Identification of marine macroalgae using PCR-RFLP and DGGE methods

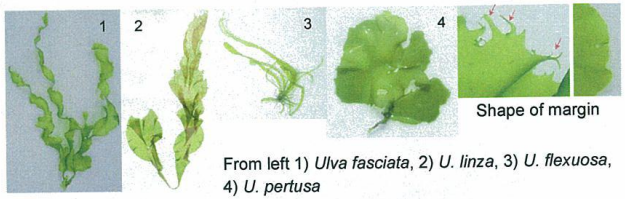
Takeaki Hanyuda (Kobe University Research Center for Inland Seas)



Identification of *Ulva* species

- 18 *Ulva* species are recognized in Japan (Yoshida et al. 2005).
- Identification of *Ulva* species using morphological characters (cell size and shape, thallus margin, number of pyrenoid, etc.) is often very difficult, because such characters are often not stable or unclear.

→ Genetic identification is necessary and effective.



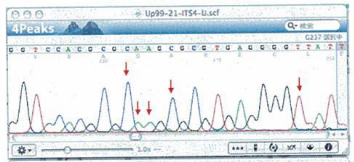
From left 1) *Ulva fasciata*, 2) *U. linza*, 3) *U. flexuosa*, 4) *U. pertusa*

Genetic identification of *Ulva* species

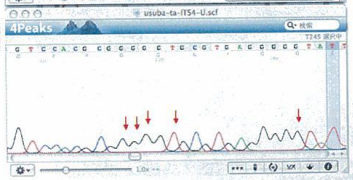
nuclear ITS (Internal Transcribed Spacer) region

Nucleotide sequences of nuclear ITS region are different among *Ulva* species, so this region is suitable for identification of *Ulva* species.

Ulva pertusa

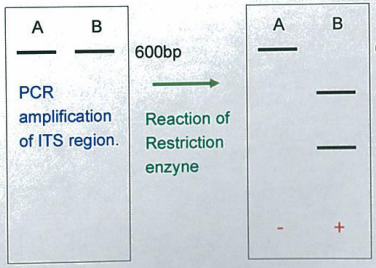


U. californica



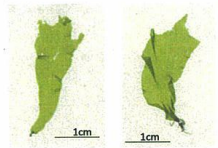
Genetic identification of *Ulva* species using PCR-RFLP method

Amplified PCR fragments are processed with restriction enzyme.
Based the on presence of restriction site, *Ulva* species are identified.

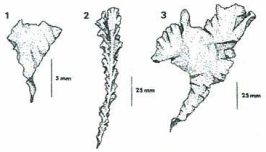


Case 1: *Ulva californica* and *U. linza*

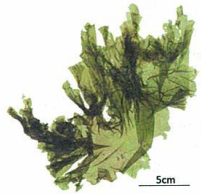
- In 2007, *Ulva californica* was first reported from Japan (Aichi and Hyogo).
- This species was assumed to be a invaded species, but it is not clear.
- Morphologically, *U. californica* is often very similar to *U. linza*.



U. californica: Tanoshiro, Hyogo



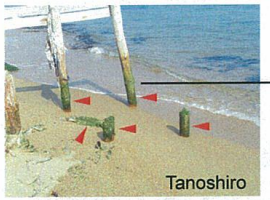
U. californica, CA, USA



U. californica: Mikawa Bay, Aichi

U. linza, Hyogo

Habitats of *U. californica* and *U. linza* in Awaji Island



Both *U. californica* and *U. linza* were collected from this part.

- In addition to morphological similarity, habitats of these two species are also similar.

- In order to distinguish *U. californica* and *U. linza*, PCR-RFLP method was employed.

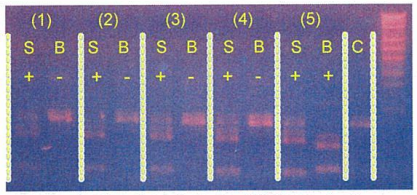
Genetic identification of *U. californica* and *U. linza* using PCR-RFLP

1) PCR amplification of ITS region

2) Reaction of restriction enzyme

	Sal I site	BamH I site
U. californica	+	-
U. linza	+	+

Sal I site: 5'-GTCGAC-3'
BamH I site: 5'-GGATCC-3'

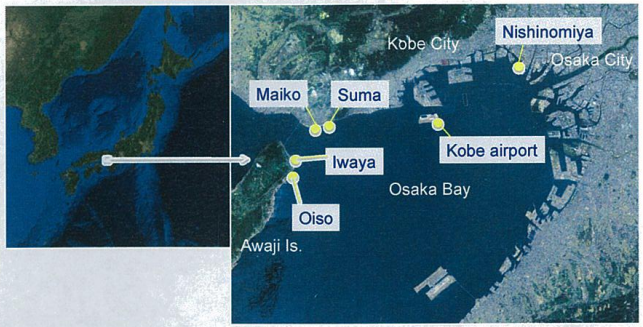


S: Sal I , B: BamH I , C: Control (PCR product)

(1) *U. californica*
(2) *U. californica*
(3) *U. californica*
(4) *U. californica*
(5) *U. linza*

U. californica and *U. linza* can be easily identified by PCR-RFLP.

Case 2: Identification of *Ulva* species in Osaka Bay



Ulva species were collected from six sites in Osaka Bay and genetically identified using PCR-RFLP method.

Restriction sites assumed from ITS sequences

	BamH I	BstE II	Sal I	Sma I	BssH II	Eco0109 I
U. arasakii	–	+	–	–	–	–
U. californica	–	–	+	+	–	++
U. compressa	–	–	–	++	+	++
U. fasciata	+	–	+	++	++	++
U. flexuosa	–	–	+	–	–	+
U. lactuca	–	–	–	–	–	–
U. linza	+	–	+	+	–	+
U. ohnoi	+	–	+	+	+	+++
U. pertusa	–	–	–	–	+	–
U. reticulata	+	–	+	+	+	+
U. rigida	+	–	+	++	+	–
U. spinulosa	+	–	+	+	+	++
U. tanneri	–	–	+	–	–	–

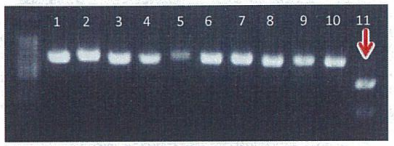
☐ *Ulva* species in Osaka Bay

+: one restriction site
++: two restriction sites
+++: three restriction sites

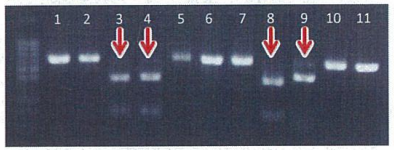
Using six restriction enzymes, 13 *Ulva* species, that are common in Japanese coast, can be identified.

A example of PCR-RFLP using *Ulva* species in Osaka Bay

1) BstE II



2) BssH II



		BstE II	BssH II
1.	*Ulva lactuca*	-	-
2.	*U. tanneri*	-	-
3.	*U. rigida*	-	+
4.	*U. pertusa*	-	+
5.	*U. ohnoi*	-	-
6.	*U. linza*	-	-
7.	*U. flexuosa*	-	-
8.	*U. fasciata*	-	+
9.	*U. compressa*	-	+
10.	*U. californica*	-	-
11.	*U. arasakii*	+	-

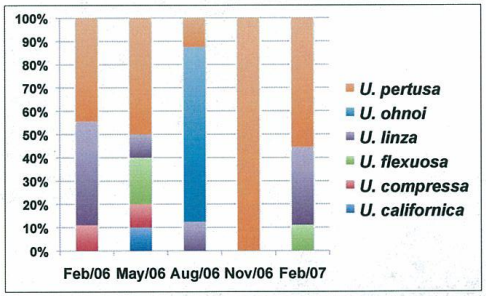
Identified *Ulva* species in Osaka Bay
(Feb 2006 - Feb 2007)

- *U. arasakii*
- *U. californica*
- *U. compressa*
- *U. fasciata*
- *U. flexuosa*
- *U. linza*
- *U. ohnoi*
- *U. pertusa*
- *U. rigida*
- *U. tanneri*

During Feb. 2006 to Feb. 2007, total 10 species were identified from six sites in Osaka bay.

Identified *Ulva* species in Osaka Bay
(Feb 2006 - Feb 2007)

Results at artificial tide-pool in Kobe airport

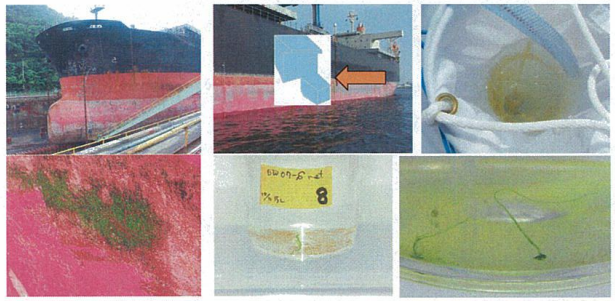


Seasonality of dominant *Ulva* species was revealed.
- *U. linza*: Winter (Feb.), - *U. pertusa*: Early winter (Nov.) to Spring (May).
- *U. ohnoi*: Summer (Aug.)

Genetic identification of *Ulva* species from environmental DNAs using DGGE method

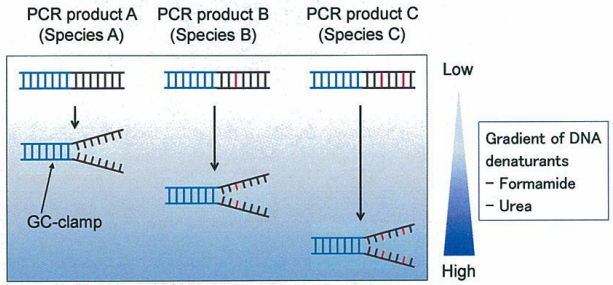
Ulva species are typical as ship hull organisms and often including in Ballast water.

→ early detection system of introduced organisms using molecular method is necessary.



Principle of DGGE analysis

In polyacrylamide gels containing a linearly increasing gradient of DNA denaturants, DNA fragments of the same length but with different base-pair sequences (at least 1 bp) were separated based on the decreased electrophoretic mobility of a partially melted DNA molecule.



Research of genetic region which is suitable for DGGE

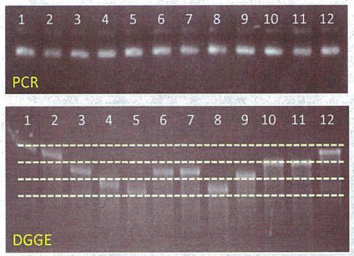
・*RbcL* gene sequences were compared among Japanese common 11 *Ulva* species.

・1-11 bp differences were shown among the portion of *rbcL* gene sequences of 11 species.

Ulva rigida	TAACCA AAGGA TTT ATTTTCTAC G T TTGTT AC
U. linza	TAGCCA GATAA TCT ACCTTTTAC A T GTGTT GT
U. arasakii	TAGTCA AATAA TTT ACTTTTGAC G G TTACT AT
U. lactuca	TAGTCA AATAA GTT ACTCTTTGC A T TCACC AT
U. fasciata	TAATGA ATGGA TTT ATTTTCTAC A T TTGTT AC
U. pertusa	TAGTCA GATAA ATC ACTTTTTAT A T TCGCT AT
U. compressa	TAGTCA AATAA TTT ACTCCTTAC A T TTGTT AT
U. californica	TAGCCG AATAA TTT ACTTTTTAC A T TTGCT AT
U. flexuosa	TAGCCA AATAA TTT ACTTTTTAC A T TTGCT AT
U. ohnoi	TAACCA ATGGA TTT ATTTTCTAC A T TTGTT AC
U. spinulosa	TAACCA ATGGA TTT GTTTTCTAC A T TTGTT AC

Design of primers for DGGE, and examination of various conditions.

Identification of Japanese *Ulva* species using DGGE



1. *Ulva rigida*
2. *U. linza*
3. *U. tanneri*
4. *U. arasakii*
5. *U. lactuca*
6. *U. fasciata*
7. *U. pertusa*
8. *U. compressa*
9. *U. californica*
10. *U. flexuosa*
11. *U. ohnoi*
12. *U. spinulosa*

Setting
6% polyacrylamide
60°C
20→50% (Gradient)
80V
16h

• PCR-DGGE indicates 7 or 8 types of DNA fragments, and 4 or 5 of them were species specific.

• It is thought that more species can be identified by a further requirement study.

Summary of genetic identification of *algae* species

- PCR-RFLP method is effective for identification of algae species.
- DGGE method seems to be suitable for identification of algal species, especially detection of introduced species is expected.

Acknowledgement

Kiyonori Cassio Nakamura (Kobe University)
Takashi Nozawa (Kobe University)

DNA barcoding of macroalgae – 'Hawaiian Rhodophyta Biodiversity Project' as a DNA-based floristic surveys

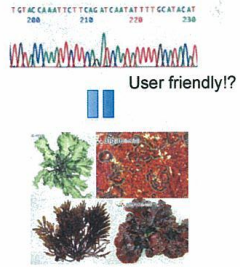
Akira Kurihara

Kobe University Research Center for Inland Seas

CAPaBLE Training Program, Kobe, December 4-12, 2011

DNA barcoding

- **DNA barcode** – short DNA sequence to be used for species identification (Hebert et al. 2003)
  - Biological universal product code analog



User friendly!?

Professional skills are required for species ID!

DNA barcoding

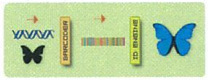
- 2003 – 648 bp near 5' end of the mitochondrial *cox*1 (COI) gene proposed as a candidate "DNA barcode"
  - Ubiquitous
  - High copy number
  - Larger divergence between animal species than many nuclear genes (for same length of DNA)
  - Large amounts of data obtainable/available, especially for animals
- First studies: animal phyla, insect orders and moth families
- First publication for macroalgae: Saunders (2005) for red algae, Evans et al. (2007) for brown algae

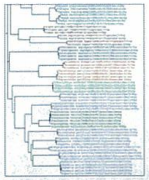
Practical aspects – how do you barcode an organism?

- Obtain 5' *cox*1 sequence for your organism
- Submit sequence via internet to **BOLD**

BOLD – "Barcode of Life Data System"

http://www.boldsystems.org/views/login.php

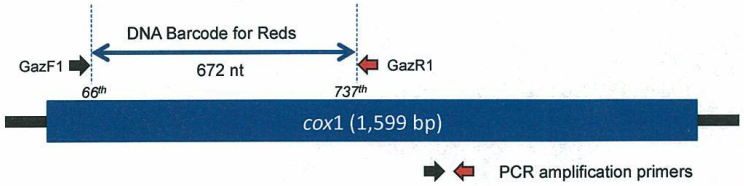




- Examine output in tree format to see nearest match to your sequence

The DNA barcode region for red algae: *cox*1

Chondrus crispus (GenBank accession: NC_001677)



Unfortunately, however, GazF1/GazR1 primer sets are not a good primer pair.
More primer sets should be tested if you get no or faint bands in PCR amplification.

Hawaiian Rhodophyta Biodiversity Survey (2006-2010)

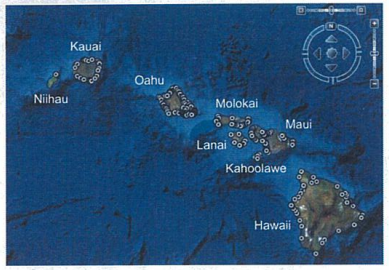
Aims to document and archive the marine, freshwater and subaerial red algae of the Hawaiian islands (vouchering morphological samples and genomic DNA extracts; to assess intraspecific variation; and to provide a framework for recognition of Hawaiian Rhodophyta molecular diversity.

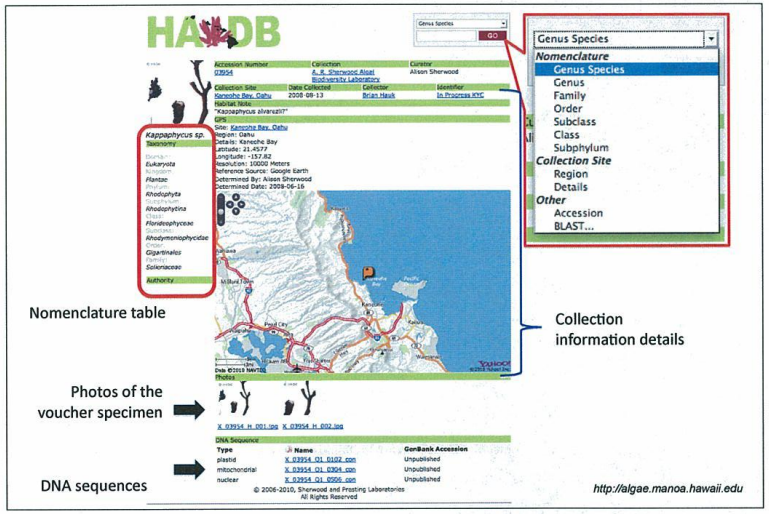
HADB
Hawaiian Algal Database

1,946 accessions

307 collection sites
MHI: 295
NWHI: 12
Marine or brackish: 292
Freshwater:13
Terrestrial: 2

2,391 sequences
28S: 904
UPA: 848
*cox*1: 639





✧ Nuclear: LSU (28S) rRNA fragment (ca. 650bp)

- Y fragment (containing a hyper variable region)
- A lack of reliable species-level resolution

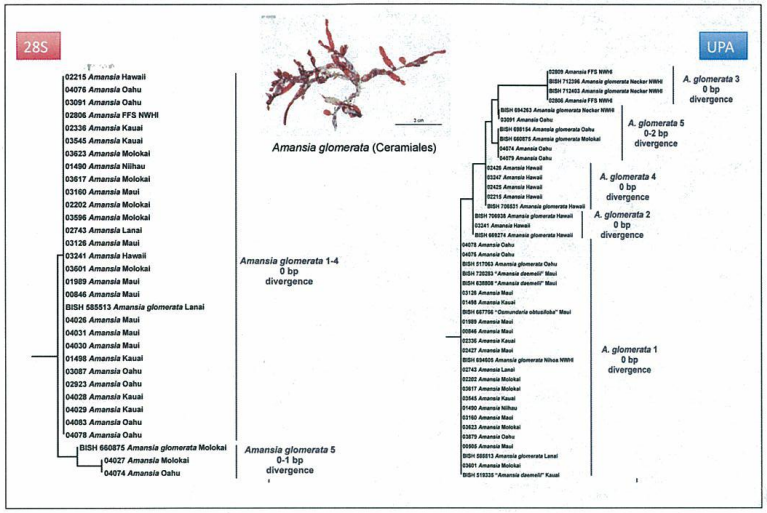
✧ Plastid: LSU (23S) rRNA fragment (ca. 410 bp)

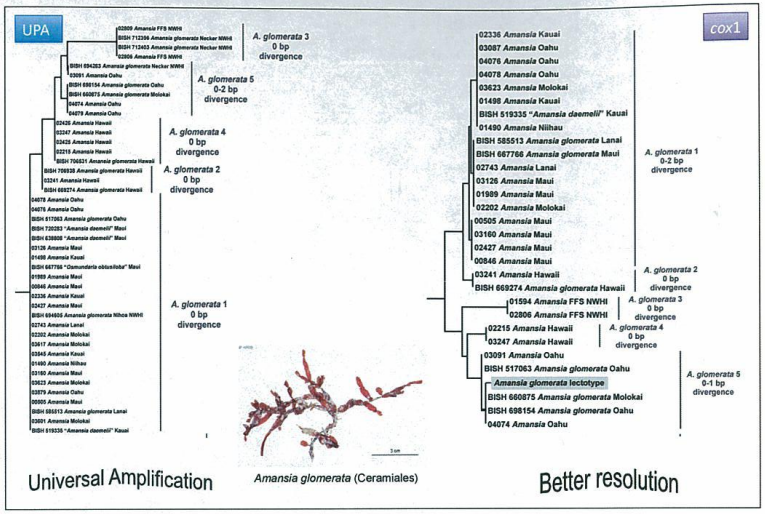
- Universal Plastid Amplicon (UPA)
- A second DNA barcode alternative
- Potentially, it can be amplified and sequenced for most cyanos and plastid-containing algal lineages (➔ higher risk of sequence contamination in comparison with *cox*1).

✧ Mitochondrion: *cox*1 fragment (ca. 650 bp)

- Lengths of the target regions are variable from primer pairs.
- No universal primer pairs have not designed so far.
  GazF1/GazR1 (Saunders 2005) for red algae
  two other reverse primers (our project)
  vs. seven combinations within a single family (Saunders' group)
- Barcode gene for red and brown algae so far.

low
Species-level resolution
high

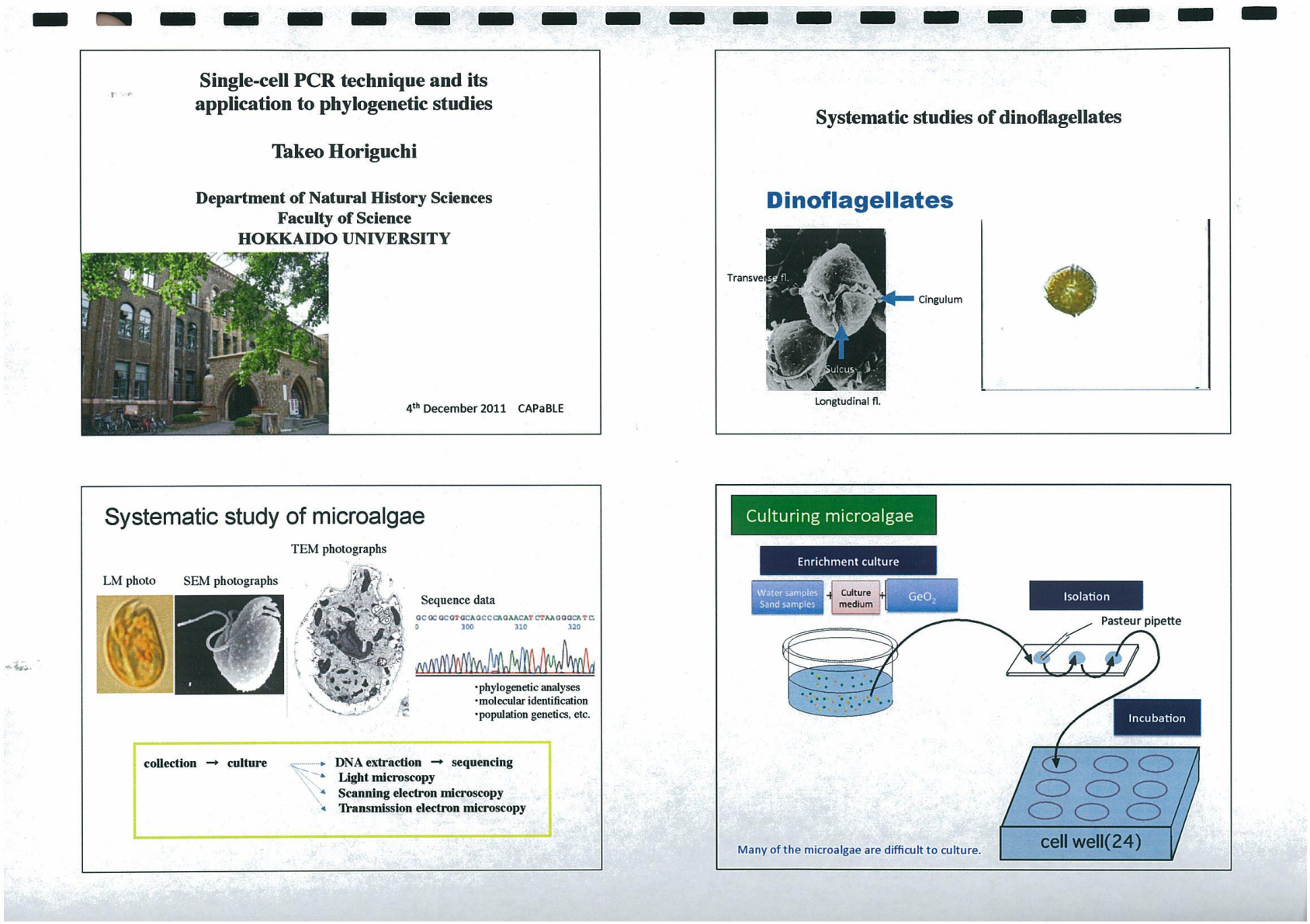




Summary – DNA barcoding

- **Advantages:**
  - may help circumvent lack of taxonomic expertise
  - more easily standardized than morphology-based taxonomy
  - can help in cases of missing life history stages and partial specimens (even degraded specimens might be okay)
  - can provide accessible species identification system to non-experts
  - demonstrates value of collections and speeds discovery of biodiversity

- **Disadvantages:**
  - reliance on a single gene risky – and *cox*1 is not the best choice for all organisms
  - may divert funding from traditional taxonomy



Single-cell PCR technique and its application to phylogenetic studies

Takeo Horiguchi

Department of Natural History Sciences
Faculty of Science
HOKKAIDO UNIVERSITY

4th December 2011 CAPaBLE

Systematic studies of dinoflagellates

Dinoflagellates

Transverse fl.
Cingulum
Sulcus
Longtudinal fl.

Systematic study of microalgae

TEM photographs

LM photo
SEM photographs

Sequence data

•phylogenetic analyses
•molecular identification
•population genetics, etc.

collection → culture
DNA extraction → sequencing
Light microscopy
Scanning electron microscopy
Transmission electron microscopy

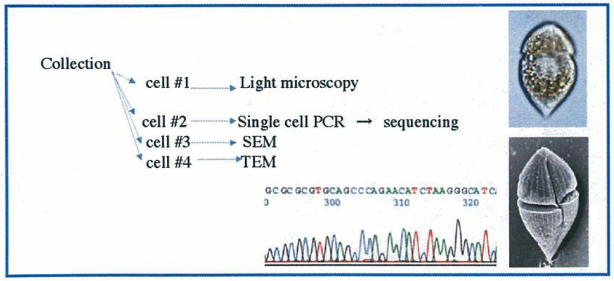
Culturing microalgae

Enrichment culture
Water samples Sand samples
Culture medium
GeO_2
Isolation
Pasteur pipette
Incubation
cell well(24)

Many of the microalgae are difficult to culture.

Obtaining both morphological and molecular data

- Without culturing・・・・using single cells from nature.
- Acquiring both morphological and molecular data at the same time.



Obtaining both morphological and molecular data

- Without culturing・・・・using single cells from nature.
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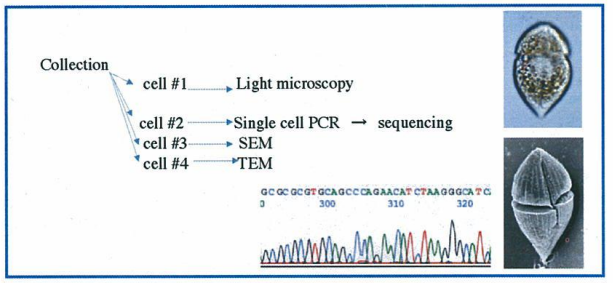
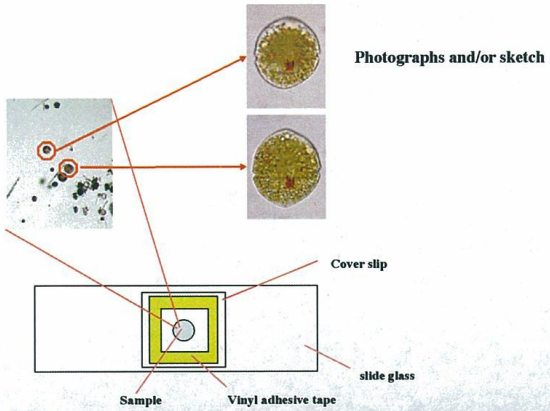
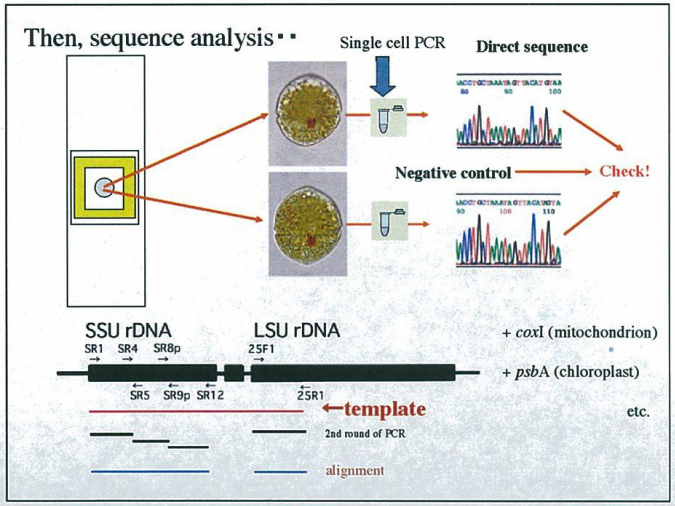


First, morphological observations・・

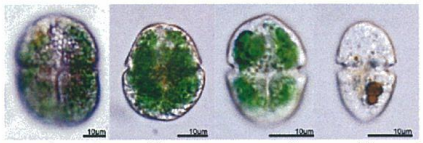


Then, sequence analysis・・



Gymnodinium aeruginosum Stein

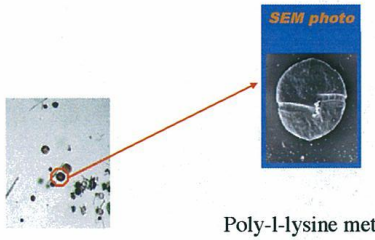
···Freshwater species



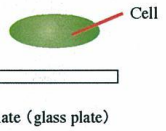
Aomori Kagawa Sapporo City centre

All of them belong to the same species!

You can take SEM photographs, too···

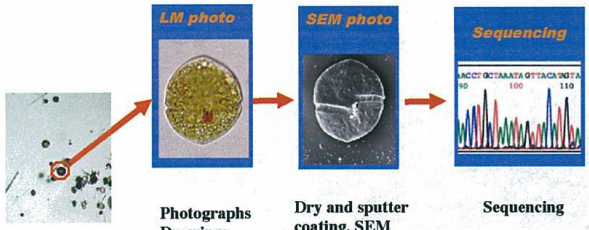


Poly-l-lysine method



● This technique can be applied to single-cell TEM sectioning!

You can obtain these data from a single cell····

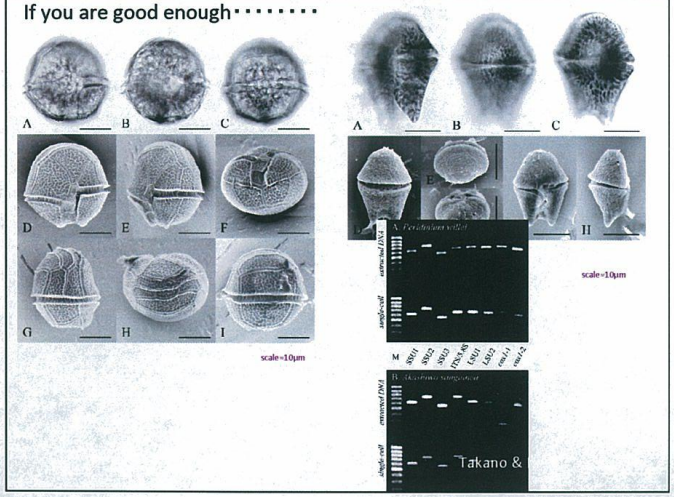


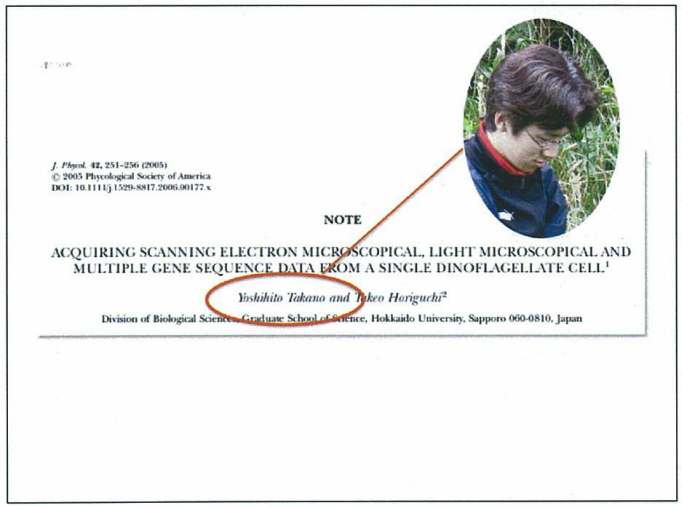
Photographs Drawings

Dry and sputter coating. SEM observations

Sequencing

If you are good enough·····





Now we use "morphological observations coupled with a single cell PCR technique" routinely.

We find this method is extremely useful to study the dinoflagellates or other protists, which are difficult to culture. Even for potentially culturable species, we can apply this method and you can speed-up biodiversity studies.

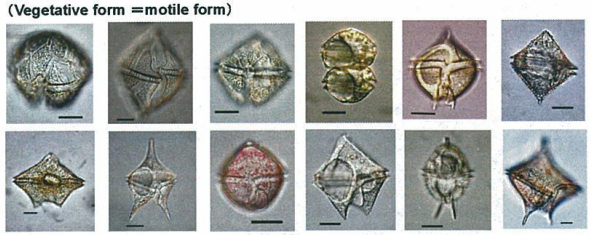
We are studying the following groups using this technique,

- freshwater dinoflagellates
- heterotrophic marine dinoflagellates, including *Protoperidinium*
- parasitic dinoflagellates
- soil protists
- other microalgae, including haptophytes

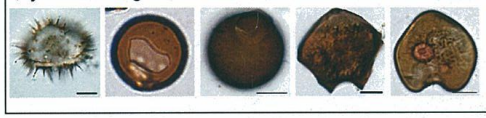
• This technique can be applicable to single-cell stage of macroalgae.

Systematic study of
heterotrophic marine dinoflagellate genus, *Protoperidinium*

(Vegetative form =motile form)

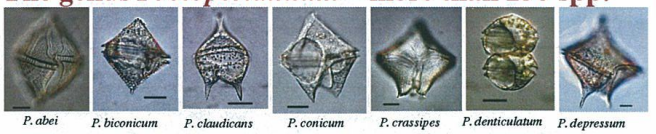


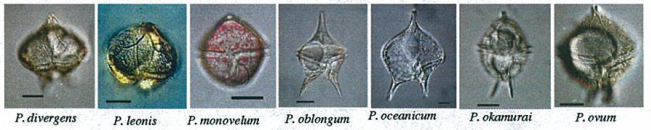
(Cyst =becoming fossil)

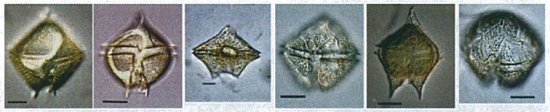


Scale bars
= 20μm

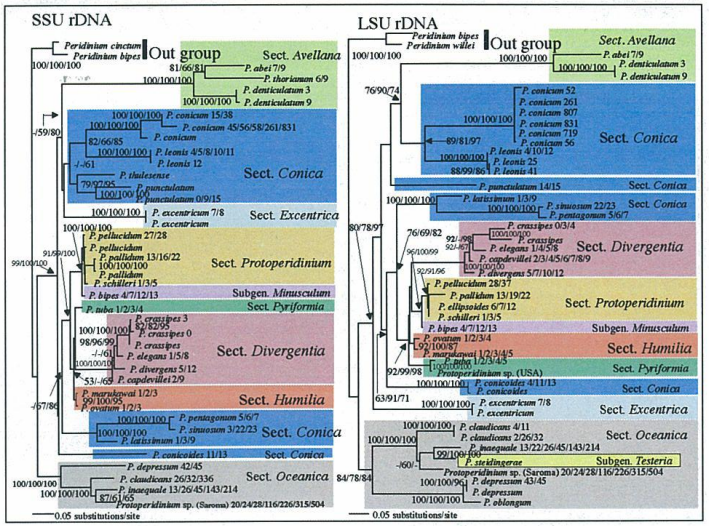
The genus *Protoperidinium* – more than 250 spp.

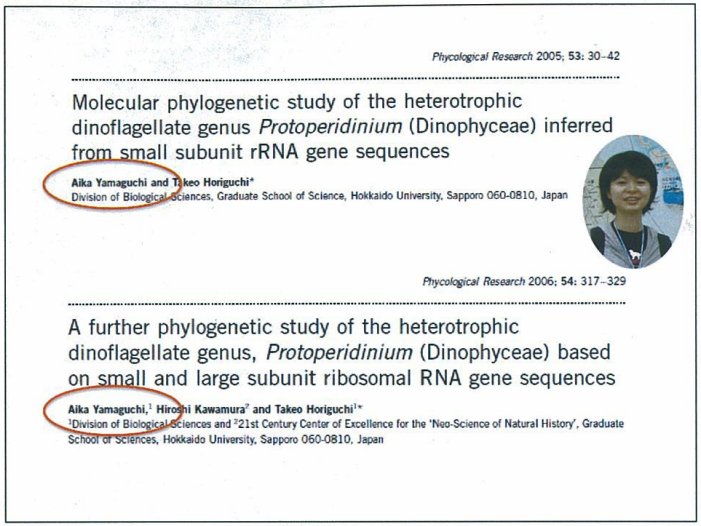






Scale bars
= 20μm





Phycological Research 2005; 53: 30–42

Molecular phylogenetic study of the heterotrophic dinoflagellate genus *Protoperidinium* (Dinophyceae) inferred from small subunit rRNA gene sequences

Aika Yamaguchi and Takeo Horiguchi*
Division of Biological Sciences, Graduate School of Science, Hokkaido University, Sapporo 060-0810, Japan

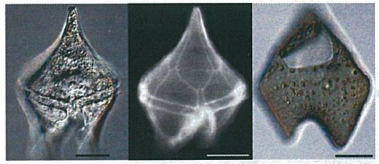
Phycological Research 2006; 54: 317–329

A further phylogenetic study of the heterotrophic dinoflagellate genus, *Protoperidinium* (Dinophyceae) based on small and large subunit ribosomal RNA gene sequences

Aika Yamaguchi,[1] Hiroshi Kawamura[2] and Takeo Horiguchi[1]*
[1]Division of Biological Sciences and [2]21st Century Center of Excellence for the 'Neo-Science of Natural History', Graduate School of Sciences, Hokkaido University, Sapporo 060-0810, Japan

A successful example:

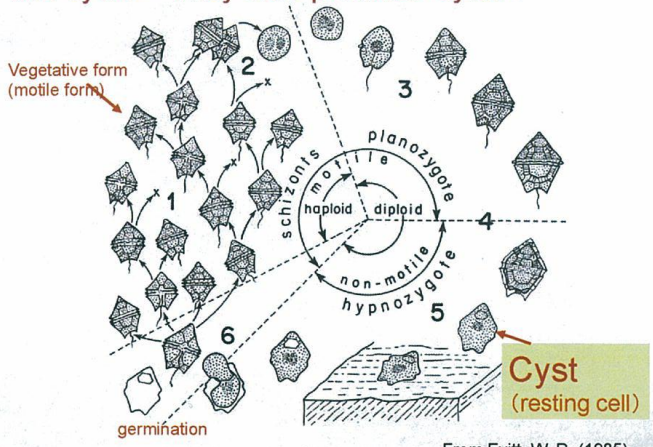
A taxonomic study of a *Protoperidinium oblongum*-complex



Scale bars; 20 μm

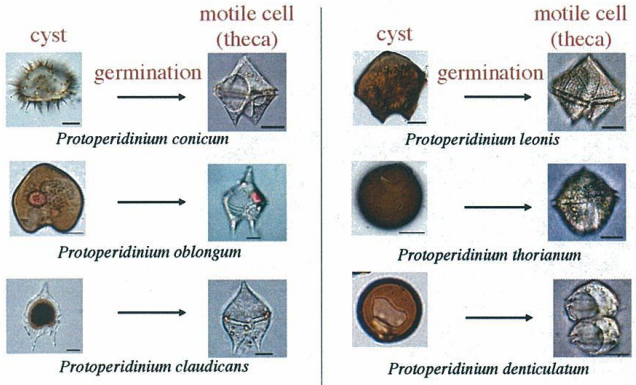
By Aika Yamaguchi, Hiroshi Kawamura and Takeo Horiguchi

Life cycle – They also produce 'cysts'.

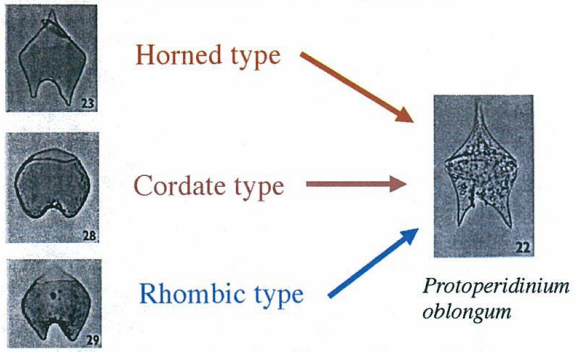


From Evitt, W. R. (1985)

Cyst-theca relationships



Three cyst types of *P. oblongum*



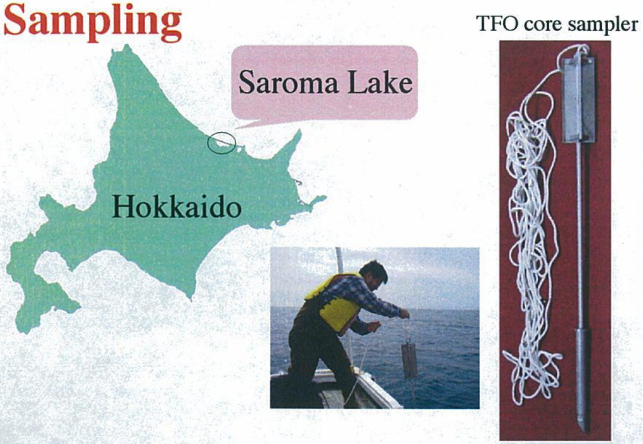
Wall, D. and Dale, B. 1968. Modern dinoflagellate cysts and evolution of the Peridiniales. *Micropalaontology* 14: 265-304.

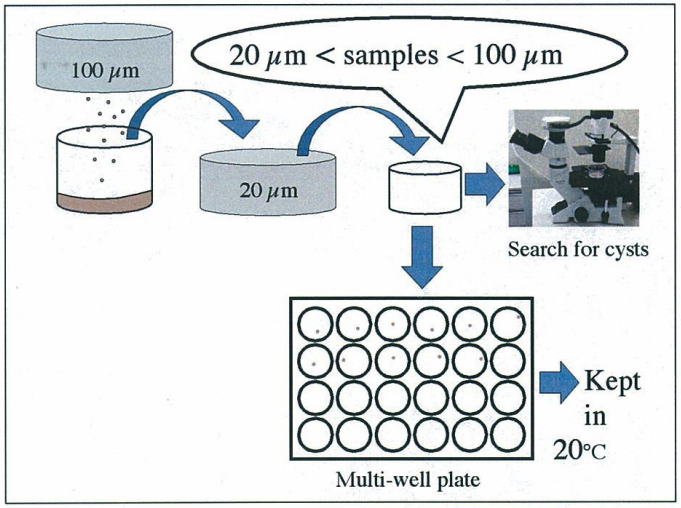
***Protoperidinium oblongum* cysts**

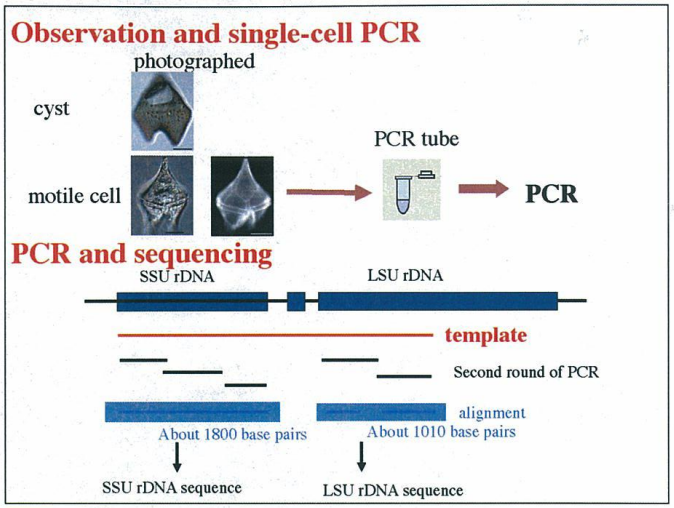
Question

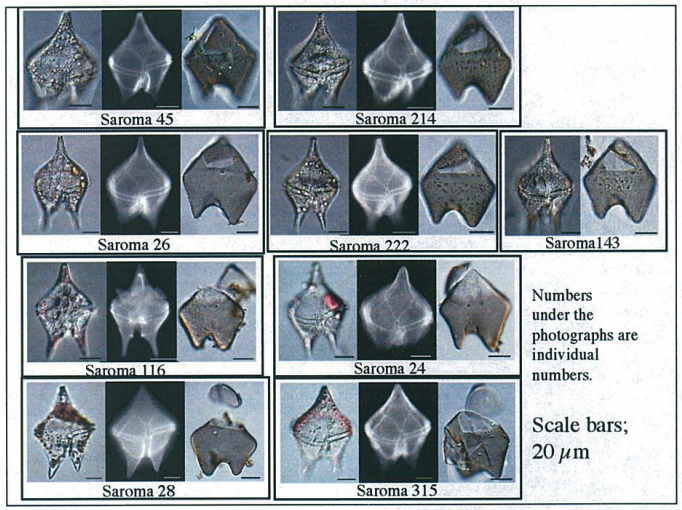
: Do these differences represent species difference or intraspecific variety?

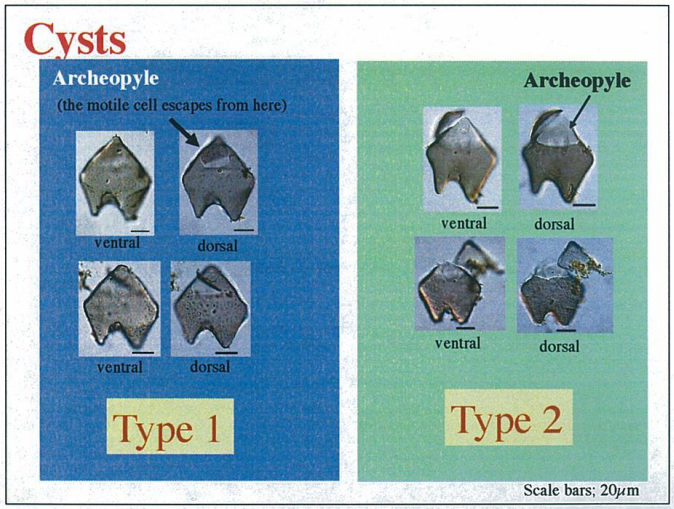
Sampling

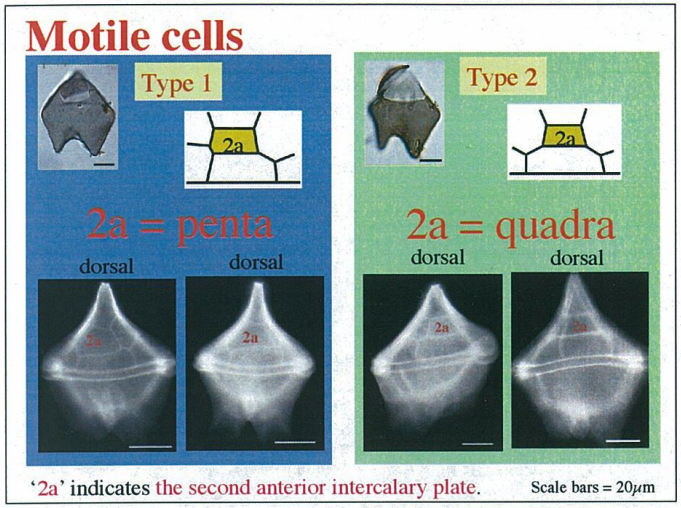


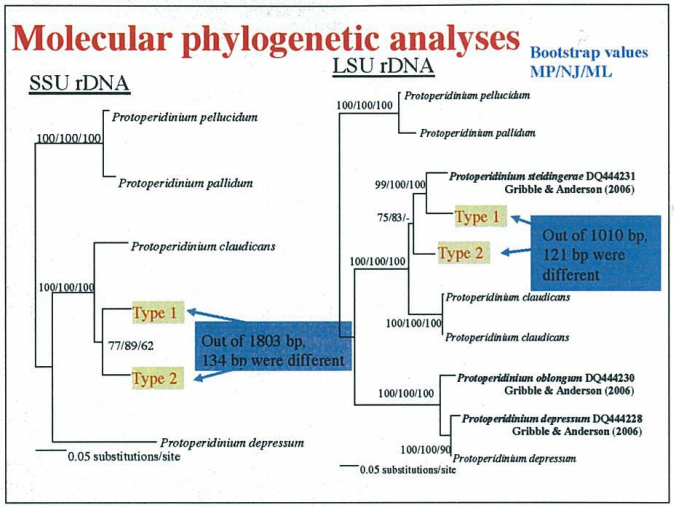

100 μm
20 μm < samples < 100 μm
20 μm
Search for cysts
Kept in 20°C
Multi-well plate

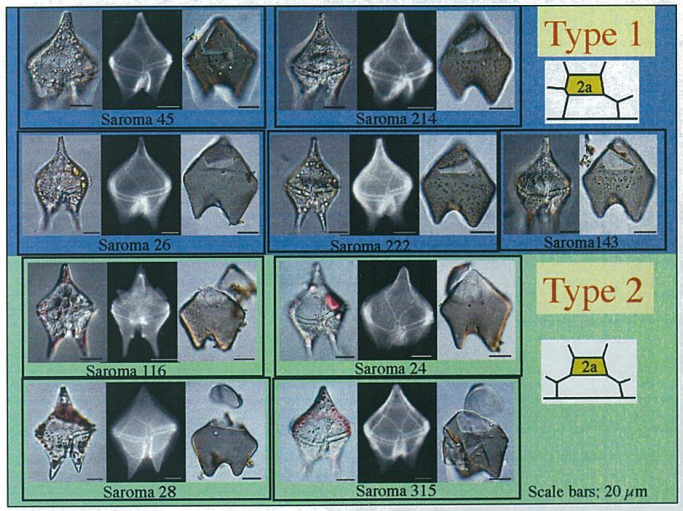

Observation and single-cell PCR
photographed
cyst
motile cell
PCR tube
PCR
PCR and sequencing
SSU rDNA
LSU rDNA
template
Second round of PCR
alignment
About 1800 base pairs
About 1010 base pairs
SSU rDNA sequence
LSU rDNA sequence

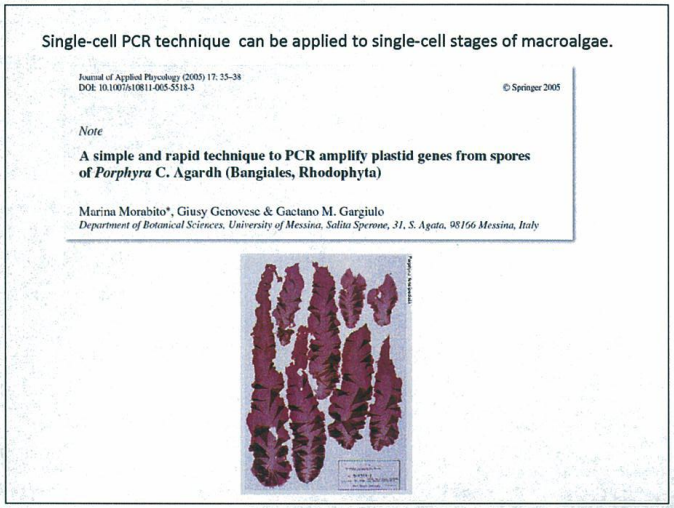

Saroma 45
Saroma 214
Saroma 26
Saroma 222
Saroma143
Saroma 116
Saroma 24
Saroma 28
Saroma 315
Numbers under the photographs are individual numbers.
Scale bars; 20 μm


Cysts
Archeopyle
(the motile cell escapes from here)
ventral
dorsal
ventral
dorsal
Type 1
Archeopyle
ventral
dorsal
ventral
dorsal
Type 2
Scale bars; 20μm

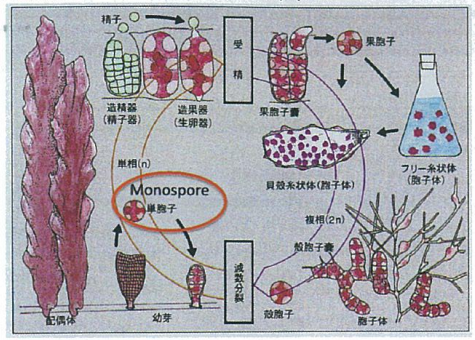

Motile cells
Type 1
2a
2a = penta
dorsal
dorsal
Type 2
2a
2a = quadra
dorsal
dorsal
'2a' indicates the second anterior intercalary plate.
Scale bars = 20μm


Molecular phylogenetic analyses
Bootstrap values
MP/NJ/ML
SSU rDNA
LSU rDNA
Protoperidinium pellucidum
Protoperidinium pallidum
Protoperidinium claudicans
Type 1
Type 2
Protoperidinium depressum
0.05 substitutions/site
Out of 1803 bp, 134 bp were different
Protoperidinium steidingerae DQ444231 Gribble & Anderson (2006)
Protoperidinium oblongum DQ444230 Gribble & Anderson (2006)
Protoperidinium depressum DQ444228 Gribble & Anderson (2006)
Out of 1010 bp, 121 bp were different


Type 1
2a
Saroma 45
Saroma 214
Saroma 26
Saroma 222
Saroma143
Type 2
2a
Saroma 116
Saroma 24
Saroma 28
Saroma 315
Scale bars: 20 μm


Single-cell PCR technique can be applied to single-cell stages of macroalgae.
Journal of Applied Phycology (2005) 17: 35–38
DOI: 10.1007/s10811-005-5518-3
© Springer 2005
Note
A simple and rapid technique to PCR amplify plastid genes from spores of Porphyra C. Agardh (Bangiales, Rhodophyta)
Marina Morabito*, Giusy Genovese & Gaetano M. Gargiulo
Department of Botanical Sciences, University of Messina, Salita Sperone, 31, S. Agata, 98166 Messina, Italy

Use of monospores as PCR template in *Porphyra*.



Use of single cells has certain advantages, such as
- No DNA extractions are required.
- Little risk of contamination.
- Can avoid difficulties, which may encounter during DNA extraction process from seaweed tissue, such as presence of copious amount of polysaccharides.

A NEW TECHNIQUE – FFPE kit

Extracting DNA from formalin-fixed, parafin-embedded tissue.

Overcoming the challenges

Around the world, scientists are taking advantage of archives of formalin-fixed, paraffin-embedded (FFPE) tissue sections to perform sophisticated molecular biology experiments. QIAGEN provides an extensive portfolio of dedicated sample and assay technologies to meet the technical challenges of working with FFPE tissues.

Challenges of working with FFPE tissues:

- Precious, irretrievable samples you cannot afford to waste
- Formalin crosslinking interfering with downstream analysis
- Highly fragmented nucleic acids
- Low DNA, RNA, and protein yields
- Potentially inaccurate genomic, transcriptomic, and proteomic data

QIAGEN

Summary

- Attempts to obtain culture strains are important and always worthwhile trying.
- If getting cultures is difficult, the single-cell PCR technique coupled with morphological observations is useful method.
- It is important to keep photomicrographic records of cells, prior to applying them to single cell PCR.
- Single cell PCR can be applied to the cells, which are even fixed, dehydrated and critical point dried for SEM observations.
- It is useful to apply multiple sets of primers for single cell PCR to obtain different DNA regions at the same time.

Appendix 6 Textbook used for Training Practices

Contents

1. Samples for DNA extraction

1) Samples dried in silica gel.
2) Samples preserved in ethanol.
3) Frozen samples
4) Pressed specimens

Samples of 1-3 are recommended for DNA extraction. It is difficult to extract a PCR-grade DNA from pressed specimens in many cases. Formalin-preserved specimens are unsuited for DNA analyses.

2. Extracting DNA

There are a lot of methods for extracting DNA from organismal tissues. For algae and plants, major methods are to use CTAB or a DNeasy Plant Mini Kit (QIAGEN, Valencia, CA, USA). The following method is modified in our laboratory from some methods and simple and low-cost.

①Place a fragment (25 mm2) of a thallus in a 1.5-ml microfuge tube.
②Add 150 µl of YK buffer*[1] and 8 µl 2-mercaptoethanol.
③Grind the fragment, using a hand-operated motor with a plastic pestle.
④Add 400µl of YK buffer, and centrifuge for 2 sec, and transfer the supernatant to a fresh tube.
⑤Centrifuge the tubes into which the supernatant was transferred at 10000 rpm for 5 min.
⑥Discard the supernatant, and add 800 µl of YK buffer and 8 µl 2-mercaptoethanol, and suspend the pellet, shaking the tube. Incubate in a water bath at 50˚C for 10 min.
⑦Centrifuge at 10000 rpm for 5 min.
⑧Discard the supernatant, and add 450 µl of HTE buffer*[2], 8 µl of 2-mercaptoethanol, 120 µl of 8M urea and 150 µl of chloroform, and suspend the pellet, shaking the tube. Mix gently for 20 min at room temperature.
⑨Add 270 µl of 3M sodium acetate and mix, and place on ice for 10 min.
⑩Centrifuge at 12000 rpm for 10 min at 4˚C.
⑪Transfer 540 µl of the supernatant to a fresh tube, and add 450 µl of isopropanol and mix gently.
⑫Centrifuge at 12000 rpm for 10 min.
⑬Discard the supernatant, and add 800 µl of 70% ethanol and mix gently.
⑭Discard the supernatant, and suspend in 100 µl of sterilized distilled water.
⑮Freeze or apply to purification with Standard Super-Cel.

CAUTION: Chloroform is harmful to both human health and the environment. It has to be trashed into a designated waste bottle.

*[1] YK buffer
10 mM Tris-HCl
10 mM Na_2EDTA
1 mM spermidine
10 mM NaCl
Adjust pH to 9.4 by 10N NaOH

*[2] HTE buffer
50 mM Tris-HCl
20 mM Na_2EDTA
Adjust pH to 7.5 by 10N NaOH

3. DNA purification with Standard Super-Cel

①Add 300 µl of 6M NaI to a tube containing 100 µl DNA solution.
②Add 15 µl of Standard Super-Cel suspension*[3] and mix gently and incubate at room temperature for 5 min. (DNA binds to the silica)
③Centrifuge in a microcentrifuge for 30 sec.
④Discard the supernatant, and add 500 µl of NEW Wash*[4], suspend the pellet, shaking the tube.
⑤Centrifuge in a microcentrifuge for 30 sec, and discard the supernatant.
⑥Wash the pellet 3 times with NEW Wash, repeating the steps 4 and 5.
⑦Add 60 µl of sterile distilled water and suspend the pellet. (DNA elutes in water)
⑧Centrifuge in a microcentrifuge for 30 sec.
⑨Transfer 50 µl of the supernatant to a fresh tube.
⑩Add 50 µl of sterile distilled water to the tube including Standard Super-Cel and suspend the pellet. centrifuge the tubes for 30 sec.
⑪Transfer 50 µl of the supernatant to the tube to which the first eluted DNA is transferred.
⑫ Freeze until required.

This cleaned DNA can be used as template for PCR.

***[3]** Standard Super-Cel (Nakalai Tesque, Inc., Kyoto, Japan) suspension
Put the powder of Standard Super-Cel into a fresh tube up to ca. 500 µl, and add 1000 µl of sterilized distilled water to wash the powder. Spin down the powder and discard the supernatant. After 3 times of washing, add 800 ml of sterilized distilled water. Use just after suspending. It **s**hould be preserved by freezing.

*[4] NEW Wash
50% EtOH
0.1M NaCl
10mM Tris-HCl
1mM EDTA
pH 7.5

4. PCR

①Prepare PCR reaction mixture.

Takara ExTaq (5 units/ml)	0.12 µl
10x Buffer	2.5 µl
dNTP mixture (2.5 mM each)	2 µl
Primer1 (20 pmol/ml)	0.25-1 µl
Primer1 (20 pmol/ml)	0.25-1 µl
Template DNA	0.5 µl
Sterilized distilled water	up to 25 µl

Follow a manufacture's instruction. When amplifying ribosomal DNA, add 1.25 µl of DMSO (5% in final concentration).

②PCR condition

94˚C for 1 min
94˚C for 20 sec
55˚C for 20 sec } 35-50 cycles
72˚C for 45 sec
72˚C for 5min

PCR condition may change depending on Taq plymerase, primers, the length of amplified DNA and a thermal cycler.

③ Run a thermal cycler and place the tubes in the equipment when the temperature becomes over 90˚C in the first denaturation. This is called a hot start method which prevent miss annealing.

④After thermal cycling is finished, freeze the tubes to preserve.

Thermal cycler

5. Agarose gel electrophoresis

Agarose electrophoresis is performed to check PCR products.

①Add 125 mg of agarose to a conical flask, and further add 12.5 ml of TAE buffer*[5], and swirl to mix.

②Put the flask into a microwave, and heat on for 1 min. Swirl the flask. If all agarose is dissolved, heat again. Caution! The flask is hot; make sure to ware a glove.

③Allow the mixture to cool for 3 min and prepare a gel tray.

④Pour the mixture into the gel tray and set a comb.

⑤Wait for 30 min to allow the gel to set.

⑥Pour a little amount of TAE on the gel, and carefully remove the comb by pulling it upwards.

⑦Place the gel with tray into an electrophoresis cell containing TAE buffer.

⑧Cut a piece of parafilm and place it on the bench top and scratch it to stick it on.

⑨Place small dots (ca 1µl) of loading buffer onto the parafilm (one dot for each PCR sample).

⑩Take 2.5 µl of a sample and mix it with the dot by pipetting up and down. Then, pipette up the mix and gently load it into the well of the gel. DNA size maker also may be loaded.

⑪Put the cell cover on and turn on the power supply. Run the electrophoresis rig for ca. 15 min at 100 volts.

⑫Turn off the power supply. Remove the gel and tray with hands gloved, and slide the gel off of the tray and put the gel into ethidium bromide solution.

⑬Soak the gel in the ethidium bromide solution for 10 min. Using a spatula, transfer the gel onto plastic wrap.

⑭Put the gel and wrap on a UV light box and turn on the UV light box.

⑮If bands are visible, take a picture.
⑯Transfer the gel and wrap into a reservoir containing bleach solution which decomposes ethidium bromide.
CAUTION: Ethidium bromide is a mutagen. Put on groves when you use the chemical.
*[5] TAE buffer

Tris	40 mM
Acetic acid	40 mM
Na_2EDTA	1 mM

Mupid Electrophoresis System

6. PEG precipitation (Purification of PCR products)
①Add 25 µl of sterilized distilled water to PCR products (22.5 µl).
②Add 30 µl of PEG-NaCl solution*[6] and mix thoroughly with a vortex mixer.
③Place tubes on ice for 1 hr.
④Centrifuge at 14000 rpm for 10 min at 4℃.
⑤Discard the supernatant carefully by pipetting. Make sure not to suck out the pellet, which is not visible.
⑥Add 150 µl of 70% ethanol.
⑦Centrifuge at 14000 rpm for 10 min at 4℃.
⑧Remove the supernatant carefully and dry up.
⑨Add 8 µl of sterilized distilled water to dissolve precipitation of DNA.
⑩Preserve at -20℃
*[6] PEG-NaCl solution:
5g PEG (polyethylene glycol #6000) and 3.65g NaCl in 25ml H2O

7. Cycle sequencing
BigDye Terminator v1.1 Cycle Sequencing Kit (Applied Biosystems) is used in this course.
①Prepare reaction mixtures of the following contents in each PCR tube. Practically prepare the reaction mixtures excluding DNA (and primers) and add DNA (and primers) to each tube after that.
1/4 Reaction Concentration and 1/2 Reaction Volume(for sequencing DNA more than 500 bp)

	(µl)
Ready Reaction Premix (2.5 x)	1
Sequencing Buffer (5 x)	1.5
Water	1.5
DMSO	0.5
Primer (1 pmol/µl)	1.6
Template DNA Solution	3.9
Total Volume	10

1/8 Reaction Concentration and 1/2 Reaction Volume(for sequencing DNA less than 500 bp)

	(µl)
Ready Reaction Premix (2.5 x)	0.5
Sequencing Buffer (5 x)	1.75
Water	1.75
DMSO	0.5
Primer (1pmol/µl)	1.6
Template DNA Solution	3.9
Total Volume	10

②Set tubes in a thermal cycler and perform cycle sequencing reaction with the following condition.

Condition of cycle sequencing

96˚C, 1 min
96˚C, 10 sec
50˚C, 5 sec } 25 cycles
60˚C, 4 min
4˚C, hold

③These products can be stored at -20˚C.

8. Purifying cycle sequencing products

Ethanol/EDTA/Sodium Acetate Precipitation

①To prepare the mixture, add followings to a fresh 1.5 ml tube and mix.

	Volume for one reaction tube
Water	10 µl
125mM EDTA	2 µl
3 M Sodium Acetate	2 µl

②Add 14 µl of the mixture to each tube containing products.
③ Add 50 µl of 95% ethanol to each tube and mix.
④Incubate at room temperature for 15 min.
⑤Centrifuge the tubes at 15000 rpm for 20 min at 6˚C.
⑥Discard the supernatant and add 150 µl of 70% ethanol.
⑦Centrifuge the tubes at 6˚C and 15000 rpm for 5 min.
⑧Discard the supernatant and dry up.
⑨These products can be stored at -20˚C.

9. Electrophoresis on ABI genetic analyzer

①Add 18 µl of Hi-Di™ formamide to each tube.
②Heat the tubes for 2 minutes at 95˚C in a thermal cycler.
③Transfer the samples to a 96-well plate, and put a plate septa on.
④Run on the sequencer.

ABI 3730 genetic analyzer

10. Aligning DNA sequences

①Align pair (forward and reverse) and continuous sequences, using a sequence editor. PAUP*, a program for phylogenetic analysis, is also useful for sequence alignment. Reverse sequences are aligned after they are converted to forward sequences. You can convert them on the Web: http://www.bioinformatics.org/sms/rev_comp.html.

②Download related sequences from DNA sequence databases: EMBL, GenBank and DDBJ. Add the downloaded sequences to your alignment. You may align by eye or using multiple alignment programs, ClustalW for example.

11. Phylogenetic analyses using PAUP

PAUP* is one of popular phylogeneitic analysis program and infers phylogenetic trees using maximum parsimony, neighbor joining and maximum likelihood methods. Purchasing information of the PAUP* can be found at http://paup.csit.fsu.edu.

PAUP* uses the NEXUS format for input data files. An example of the NEXUS format is below.

```
#NEXUS
Begin data;
Dimensions ntax = 5 nchar = 10;
Format datatype = nucleotide gap = - missing = N;
Matrix
A001   ATGCTTACGATC
A002   ATCCTT-CGATC
B001   ATCGTAACGATC
B002   ATGCTAACGATC
C001   ATAATTACGTTN
;
END;
```

Maximum parsimony analysis by PAUP.

①Run PAUP and execute a file. PAUP uses the NEXUS format for input data files.

②Type "outgroup *** *** (OTU label)" to specify taxa as an outgroup.

③Type "BandB" in the command line and run: branch and bound search is performed.

④Type "showtree 1" to show the inferred tree.

⑤Type "savetrees file=*.tre brlen=yes from=1 to=1" to save the tree with branch lengths.

⑥Type "bootstrap nrep=100 search=heuristic" to perform bootstrap analysis.

⑦Type "savetrees file=*boot.tre savebootp=nodelabels from=1 to=1

⑧Run TreeView program and open the saved tree file. Print trees.

12. Culturing microalgae

The text below indicates the standard method of culturing microalgae in our laboratory. For the purpose of our studies, the axenic (bacteria-free) cultures are not required and therefore, the following procedure does not include how to establish axenic cultures.

12-1 Enrichment culture

When the number of cells you wish to culture is too small, it is necessary to process your sample by enrichment culture first. Place certain amount of seawater sample or a spoonful of sand sample in a plastic cup. Add approximately the same volume (or more) of culture medium (e.g. Provsoli's PES medium, Guillard's f/2 medium or IMK medium). To prevent growth of diatoms, 10 mg/l GeO2 should be added (If you wish to culture diatoms, make sure not to add GeO2 !). Place the plastic cup under high light intensity (e.g. 50 µmol/m2/s) in 15-25 □C (the temperature is different, depending on the origin of the sample – if the sample is from temperate region, place the cup in 15-20 □C, while if the sample is from tropical, then place the cup in 25 □C) and wait for a few days. Observe this

culture everyday using an inverted microscope for appearance of any interesting algae. If you find

interesting algae, then proceed to isolation technique.

12-2 Isolation of microalgae

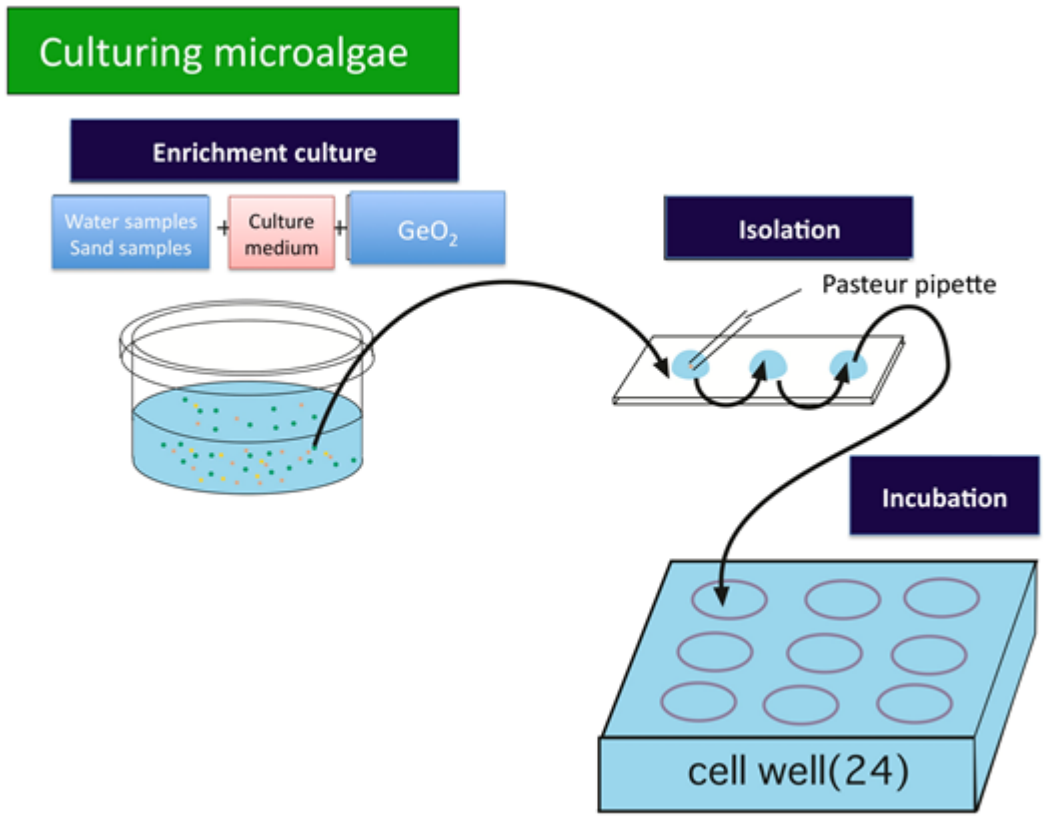
①Preparation of capillary pipette: Heat the tip of capillary pipette in the flame of alcohol lamp. When the glass becomes soft, draw the tip of the pipette with forceps and make tip of pipette very fine needle. Prepare the same capillary pipette at least three.

②Connect capillary pipette at one end of silicone tube and put the other side of the tube into your mouth- you use the tube to brow the cell out of capillary pipette.

③Observe the sample under an inverted microscope and look for the cell that you wish to isolate. When you spot the cell, bring the tip of capillary pipette to near the cell. The cell will be withdrawn into the pipette with capillarity force. It is difficult to isolate only the cell you are up to and other species of algae or debris might be sucked up together. But do not worry.

④Transfer the content of the capillary pipette into a drop of sterilized medium on a glass slide or specialized depression slide. Use a new pipette and once again look for the cell in the medium and suck it up with the capillary pipette. Then transfer the content into a new drop of fresh medium. Repeat this procedure until the drop of medium contains only the cell you wish to isolate.

⑤Once again, change the capillary with a new one. Isolate the cell and transfer the cell in a well of cell well plate. After finishing all the isolation, seal the plate and place it in the culture cabinet.



Outline of culturing microalgae.

13. Single cell PCR technique using FFPE kit

13-1. Morphological observation

①Adhere a piece of sticky tape onto a slide glass. Cut out inside of the tape with blade square and make a square frame. Isolate the cell you wish to observe and transfer the cell in the centre of the frame and seal it with a cover slip.

② Observe the cell under high magnification and take photographs of good quality.

13-2. Preparation of FFPE kit

①Repeated freezing – thawing of the solution is not good for the chemical. It is recommended that each 10 µl of FFPE solution is put into a PCR tube and is kept in a deep freezer (-20 ⍰C).

13-3. Single cell PCR using FFPE kit

①After observation and taking photographs, remove a cover slip carefully and recover the cell with a capillary pipette under the microscope and transfer the cell in a PCR tube containing 10 µl of FFPE solution (see above).

②Use thermal cycler and incubate the tube at 56 ⍰C for 1 hour, then 98 ⍰C for 2 min. This solution can be used as a DNA template for the PCR. Perform PCR as described in section 4.

③For details of FFPE kit, please see the information below.

The following information was obtained from information sheet provided by Epicentre® at Illumina® company.

QuickExtract™ FFPE DNA Extraction Kit

The QuickExtract™ FFPE DNA Extraction Kit for formalin-fixed paraffin-embedded (FFPE) tissue provides a fast, simple, and inexpensive method for preparing genomic DNA for PCR amplification from archival samples. QuickExtract DNA Extraction requires only heat treatment to melt the paraffin, lyse the cells, decrease the formalin-induced cross-linking in the sample, and degrade compounds that may inhibit amplification. Following heat treatment, the sample DNA is ready for PCR.

Product specifications

Storage: Store the QuickExtract FFPE DNA Extraction Solution at –20°C in a freezer without a defrost cycle. Minimize the number of freeze/thaw cycles. Thawed extraction solution can be stored at 4°C for 1 month or refrozen in small aliquots.

Quality Control: The QuickExtract FFPE DNA Extraction Kit is function-tested by assaying for a PCR product from DNA extracted from a slide-mounted, FFPE tissue slice.

Contaminating Activity Assays: The QuickExtract FFPE DNA Extraction Solution is free of detectable RNase, exonuclease, and endonuclease activities.

Notes on Use of the QuickExtract FFPE DNA Extraction Kit

①The yield of extracted DNA will vary by tissue type, size, and preservation methods. Approximately 1-2 μ g of DNA is obtained per square centimeter of tissue section.

②Nucleic acids isolated from preserved, paraffin-embedded tissues are generally of poor quality. The degree of degradation of these samples limits analysis to mainly techniques involving amplification.

A. FFPE Tissue Slices from Microscope SlidesÄb0

①Add 100 μ l of QuickExtract FFPE DNA Extraction Solution to the paraffin-embedded tissue section on the slide (0.8-1.0 cmÄsl181Äslmult0 2 tissue section). Scrape with a sterile blade to remove the tissue section from the slide and transfer the solution and tissue to a small microcentrifuge tube. Alternatively, the tissue section can be scraped and added to the solution in the tube, but prewetting the slide facilitates transfer of the tissue slice.

Note: If using a larger or smaller amount of tissue, adjust the reagent volume accordingly.

②Briefly centrifuge the tube to collect the solution and tissue at the bottom of the tube. If some tissue remains on the wall of the tube, begin heating the sample (Part A, Step 3) to melt the paraffin, then mix by vortexing and briefly centrifuge to collect the melted sample at the bottom of the tube.

③Heat the tube in a thermocycler for 60 minutes at 56°C, and then for 2 minutes at 98°C.

If desired, mix the sample by vortexing once during the incubation to aid in extraction, then briefly centrifuge the sample and continue the incubation.

④Quantitate the DNA yield by fluorimetry using Hoechst dye 33258 to avoid an overestimation given by A260 readings.

⑤Store the DNA at –20°C, or at –70°C for archival purposes.

B. Paraffin-Embedded Tissues

①Remove a section of tissue using a clean microtome blade. Trim off any excess paraffin.

②Place 10-50 mg of tissue or up to three 5-10 μm thick paraffin sections into a small microcentrifuge tube containing 100 μl of QuickExtract FFPE DNA Extraction Solution.
Note: The amount of extraction solution used can be adjusted to produce more concentrated extracted DNA. Thin slices are more important than the amount of tissue.

③Follow Part A, Steps 2 through 5 of the FFPE Tissue Slices from Microscope Slides protocol (above).

PCR Amplification Recommendations

①1-10 μl of extracted DNA can be used directly in standard and fast end-point PCR cycling profiles. Profiles should include 40 amplification cycles to ensure amplification.

②Primers should be designed so that PCR amplicons will be less than 300 bases in length. The average size DNA that is extracted from FFPE tissues has been reported as 300-400 bp. Real-time PCR amplicons should be less than 200 bp in length.

③Extracted DNA has been used successfully in standard and fast end-point PCR, random amplification of polymorphic DNA (RAPD) PCR, mitochondrial PCR, and real-time PCR. The resulting amplicons can be used for single-nucleotide polymorphism (SNP) detection or DNA sequencing.

References

1. Godfrey, T.I. et al.(2000) J. Mol. 2, 84.
2. Lehmann, U. and Kreipe, H. (2001) Methods 25, 409.

14. Single cell PCR technique without using FFPE kit (Conventional method)

14-1. Morphological observation

①Adhere a piece of sticky tape onto a slide glass. Cut out inside of the tape with blade square and make a square frame. Isolate the cell you wish to observe and transfer the cell in the centre of the frame and seal it with a cover slip.

②Observe the cell under high magnification and take photographs of good quality.

14-2. Recovery of cell

①Remove the cover slip carefully and recover the cell using a capillary pipette. Transfer the cell into a drop of sterilized distilled water. Waite several minutes for the cell to rupture because of osmotic pressure. If the cell does not rupture at this stage, try to break the cell using a fine glass stick – breaking a cell prior to PCR is important way to successful PCR! Suck the broken cell together with small amount of distilled water and use it as a DNA template for the PCR.

14-3. Rest of the procedure for the PCR is same as described in section 4.

Reference

Takano, Y. and Horiguchi, T. (2006) Acquiring scanning electron microscopical, light microscopical and multiple gene sequence data from a single dinoflagellate cell. J. Phycol. 42: 251-256.

15. Plant Genomic DNA Extraction using CTAB

Materials

CTAB buffer
Microfuge tubes
Mortar and Pestle
Liquid Nitrogen
Microfuge
Absolute Ethanol (ice cold)
70% Ethanol (ice cold)
7.5 M Ammonium Acetate

55℃ water bath

Chloroform : Iso Amyl Alcohol (24:1)
Water (sterile)
CTAB buffer 100ml
2.0 g CTAB (Hexadecyl trimethyl-ammonium bromide)
10.0 ml 1 M Tris pH 8.0
4.0 ml 0.5 M EDTA pH 8.0 (EthylenediaminetetraAcetic acid Di-sodium salt)
28.0 ml 5 M NaCl
40.0 ml H2O
1 g PVP 40 (polyvinyl pyrrolidone (vinylpyrrolidine homopolymer) Mw 40,000)
Adjust all to pH 5.0 with HCL and make up to 100 ml with H2O.

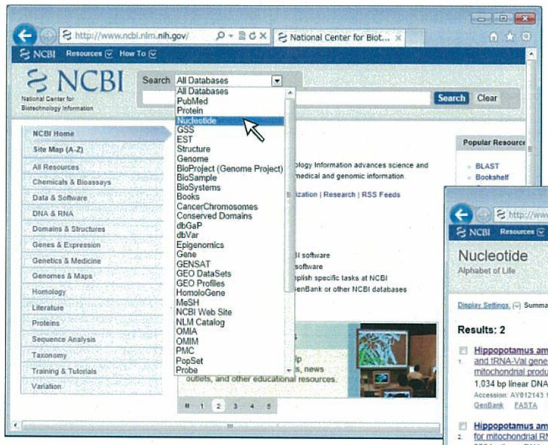
Procedure

① Grind 200 mg of plant tissue to a fine paste in approximately 500 μl of CTAB buffer.
②Transfer CTAB/plant extract mixture to a microfuge tube.
③Incubate the CTAB/plant extract mixture for about 15 min at 55oC in a recirculating water bath.
④After incubation, spin the CTAB/plant extract mixture at 12000 g for 5 min to spin down cell debris. Transfer the supernatant to clean microfuge tubes.
⑤To each tube add 250 μl of Chloroform : Iso Amyl Alcohol (24:1) and mix the solution by inversion. After mixing, spin the tubes at 13000 rpm for 1 min.
⑥Transfer the upper aqueous phase only (contains the DNA) to a clean microfuge tube.
⑦To each tube add 50 μl of 7.5 M Ammonium Acetate followed by 500 μl of ice cold absolute ethanol.
⑧Invert the tubes slowly several times to precipitate the DNA. Generally the DNA can be seen to precipitate out of solution. Alternatively the tubes can be placed for 1 hr at 20℃ after the addition of ethanol to precipitate the DNA.
⑨Following precipitation, the DNA can be pipetted off by slowly rotating/spinning a tip in the cold solution. The precipitated DNA sticks to the pipette and is visible as a clear thick precipitate. To wash the DNA, transfer the precipitate into a microfuge tube containing 500 μl of ice cold 70% ethanol and slowly invert the tube. Repeat. (alternatively the precipitate can be isolated by spinning the tube at 13000 rpm for a minute to form a pellet. Remove the supernatant and wash the DNA pellet by adding two changes of ice cold 70% ethanol).
⑩After the wash, spin the DNA into a pellet by centrifuging at 13000 rpm for 1 min. Remove all the supernatant and allow the DNA pellet to dry (approximately 15 min). Do not allow the DNA to over dry or it will be hard to re-dissolve.
⑪Resuspend the DNA in sterile DNase free water (approximately 50-400 μl H2O; the amount of water needed to dissolve the DNA can vary, depending on how much is isolated). RNaseA (10 μg/ml) can be added to the water prior to dissolving the DNA to remove any RNA in the preparation (10 μl RNaseA in 10ml H_2O).
⑫After resuspension, the DNA is incubated at 65oC for 20 min to destroy any DNases that may be present and store at 4℃.
⑬ Agarose gel electrophoresis of the DNA will show the integrity of the DNA, while spectrophotometry will give an indication of the concentration and cleanliness.

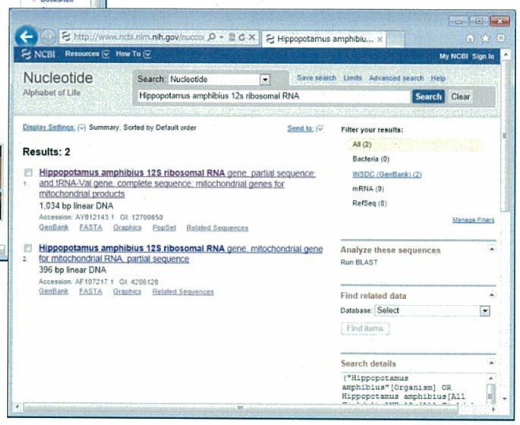
16. Construct molecular Phylogenetic Trees

Introduction to construct molecular phylogenetic trees

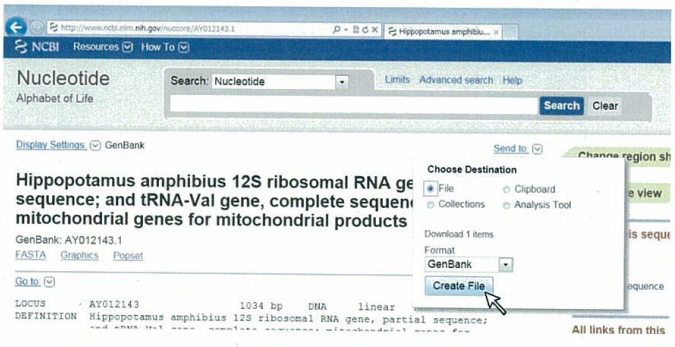
Mitsunobu Kamiya (Fukui Prefectural University)



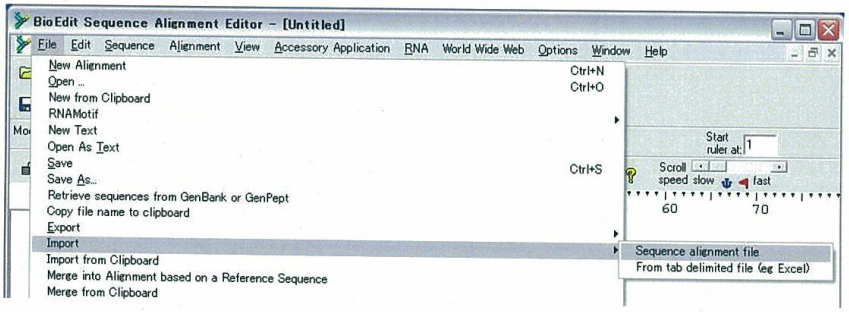
You can download any sequence data from National Center for Biotechnology Information (NCBI) web site . Choose "Nucleotide" from "Search" menu and enter an organism name & a gene name, or an accession number.



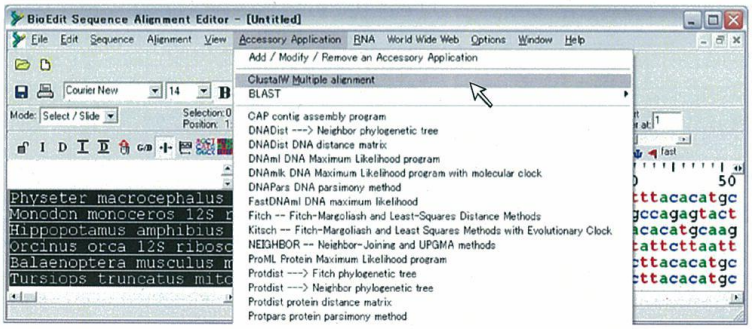
If multiple candidates are hit, select the datum including the longest sequence.



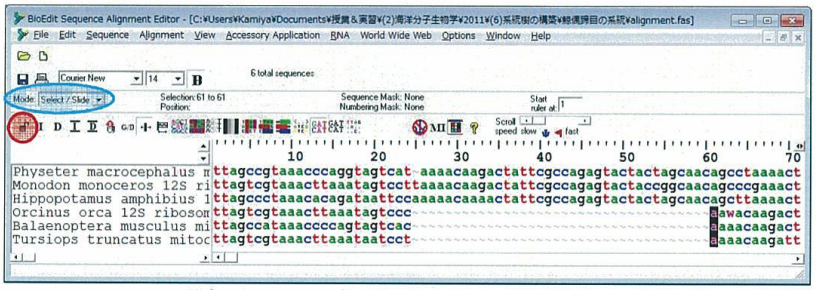
When you download the sequence datum, select "File" in "Choose Destination" and "GenBank" in "Format" from "Send to" menu. Click "Create File" and save the file wherever you want.



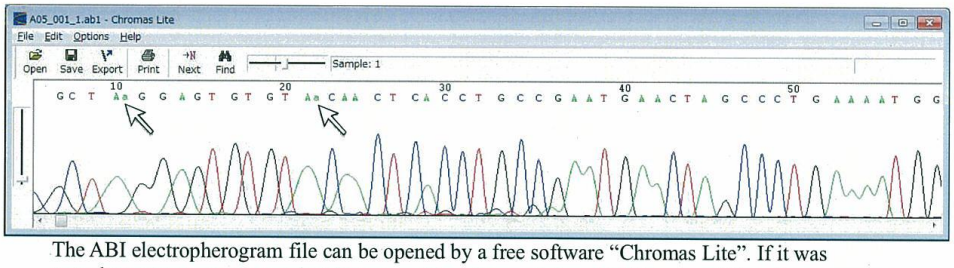
Open Bioedit Sequence Alignment Editor and select "New Alignment" from "File" menu. Import the sequence data you downloaded from "Sequence alignment file" ("File" → "Import").



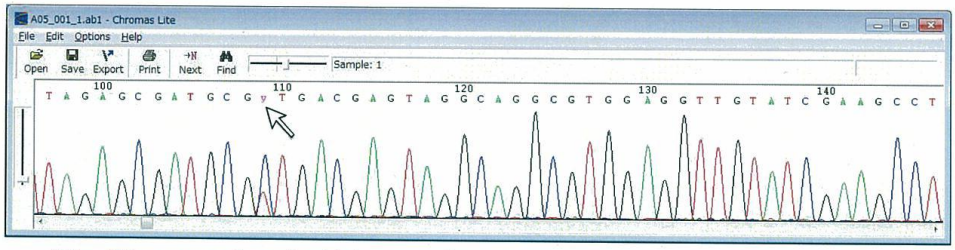
If you align the data, select all of the OTU names and select "ClustalW Multiple alignment" from "Accessory Application" menu. Click "Run ClustalW".



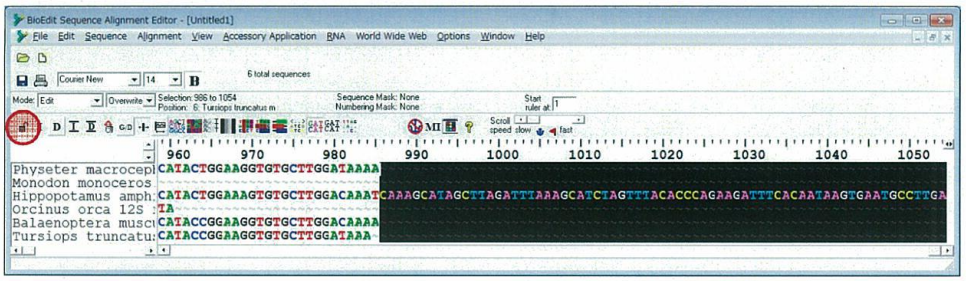
When you manually align the data, you can drag the sequences once the key icon (red circle) is clicked twice. You can modify the sequence data by selecting "Edit" from "Mode" menu (blue circle).



The ABI electropherogram file can be opened by a free software "Chromas Lite". If it was complementary sequence, select "Reverse+Complement" from "Edit" menu. When the sequences are obviously contradictory to the electropherogram pattern, the sequence should be edited. In the above case, "a" are added at the 11th and 22nd sites, respectively. In the below case, the double peaks of "c" and "t" are represented as "y" at the 109th site.

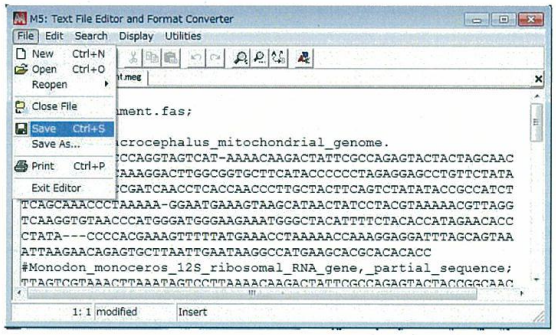


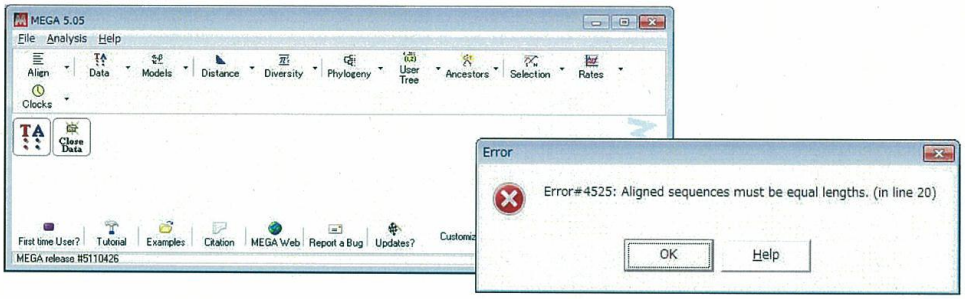
When this sequence datum is added to your alignment file, copy the sequence data to clipboard ("Edit" → "Copy Sequence" → "FASTA format"). Back to the alignment file and import the sequence datum by selecting "Import from Clipboard" from "File" menu.



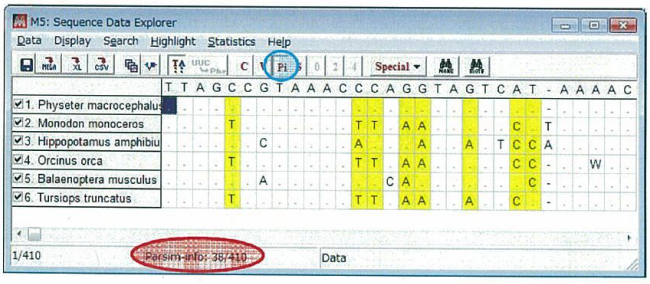
Before saving, the following matters should be checked for the further phylogenetic analysis: 1) delete the excessive sequences or add gaps to adjust the sequence length between the OTUs; 2) Do not use any symbols, including space, hyphen, slash or parentheses, for OTU names (underscore "_" is OK); 3) the gap marks should be changed to hyphen, not wave dash "~", by clicking Key icon (red circle). Finally save the file as Fasta format.

Open MEGA5 and select "Convert To MEGA Format" from "File" menu. Choose the alignment fasta file. Then save the MEGA format file by selecting "Save" from "File" menu.

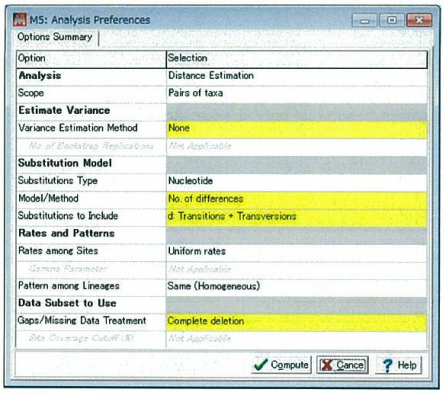




Return to the main window of MEGA5 and open the MEGA format file. Select "Nucleotide Sequences" in "Data Type". If successful, the two icons appear in the main window (the upper left figure). If an error window appears, you have to revise the alignment file according to the error message (the upper right figure).

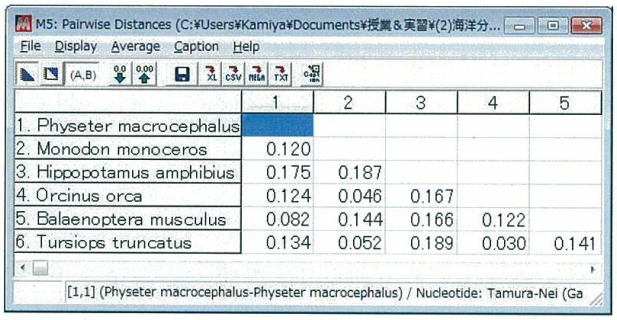


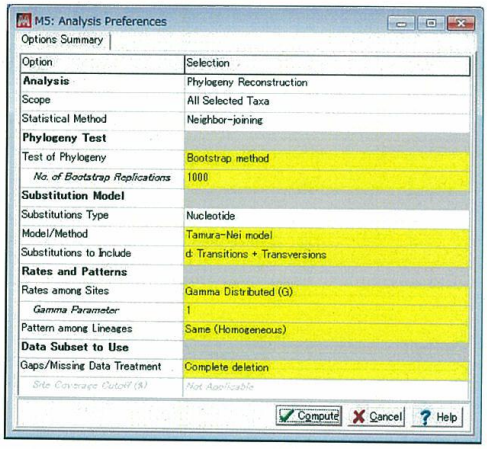
The sequence data can be seen by opening Sequence Data Explorer (the left icon). Rate of phylogenetic informative sites (red circle) can be seen by clicking Pi button (blue circle).



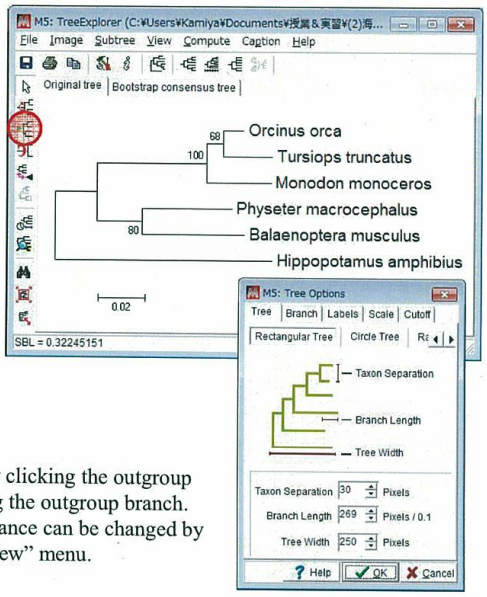
To check the number of different sites between the OTUs, select "Compute Pairwise Distances" from "Distance" icon and choose "No. of differences" from "Model/Method" option. If you remove the indel sites from the analysis, choose "Complete deletion" from "Gaps/Missing Data Treatment".

To check the genetic distances between the OTUs, choose an appropriate genetic distance model from "Model/Method" option. Neighbor-Joining tree is constructed based on these genetic distances.





Phylogenetic trees can be obtained from "Phylogeny" menu. Number of bootstrap replications, substitution model and gap/missing data treatment can be changed in the left window.



Outgroup can be specified by clicking the outgroup icon (red circle) and selecting the outgroup branch. Tree arrangement and appearance can be changed by selecting "Options" from "View" menu.

17. Appendix

Information of primers used in this course

Primers to amplify the psaA and psbA genes.

For psaA gene

psaA130F	AACWACWACTTGGATTTGGAA
psaA870F	GGNGGWYTATGGTTAAGTGA
psaA970R	GCYTCTARAATYTCTTTCA
psaA1760R	CCTCTWCCWGGWCCATCRCAWGG

For psbA gene

psbA-F	ATGACTGCTACTTTAGAAAGACG
psbA500F	CTCTGATGGWATGCCWYTAGG
psbA600R	CCAAATACACCAGCAACACC
psbA-R2	TCATGCATWACTTCCATACCTA

(The first PCR with the pair of psbA-F and psbA-R2 is followed by the second PCR with the pair of psbA-F and psbA600R or the pair of psbA500F and psbA-R2.)

These primers were designed by: Yoon et al. 2002. A single origin of the peridinin-and fucoxanthin-containing plastids in dinoflagellates through tertiary endosymbiosis. Proc. Natl. Acad. Sci. USA 99: 11724-9.

Primers to amplify the ITS2 region of ribosomal DNA.

5.8SBF (Forward)	CGATGAAGAACGCAGCGAAATGCGAT
AB28 (Reverse)	GGGATCCATATGCTTAAGTTCAGCGGGT

These primers were designed by: Goff et al. 1994. J. Phycol. 30: 521-537

Primers to amplify the small subunit ribosomal RNA gene.

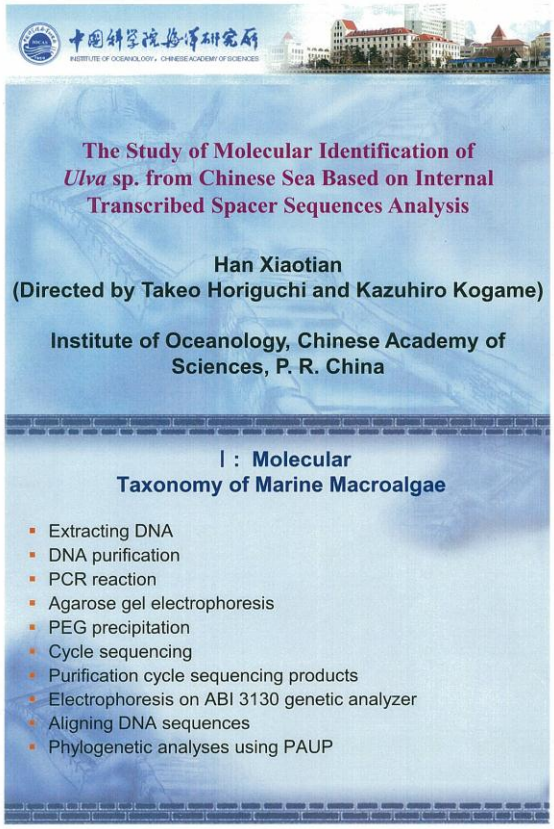
SR1 (Forward)	TACCTGGTTGATCCTGCCAG
SR4 (Forward)	AGGGCAAGTCTGGTGCCAG
SR5 (Reverse)	ACTACGAGCTTTTTAACTGC
SR8 (Forward)	GGATTGACAGATTGAGAGCT
SR9 (Reverse)	AACTAAGAACGGCCATGCAC
SR12 (Reverse)	CCTTCCGCAGGTTCACCTAC

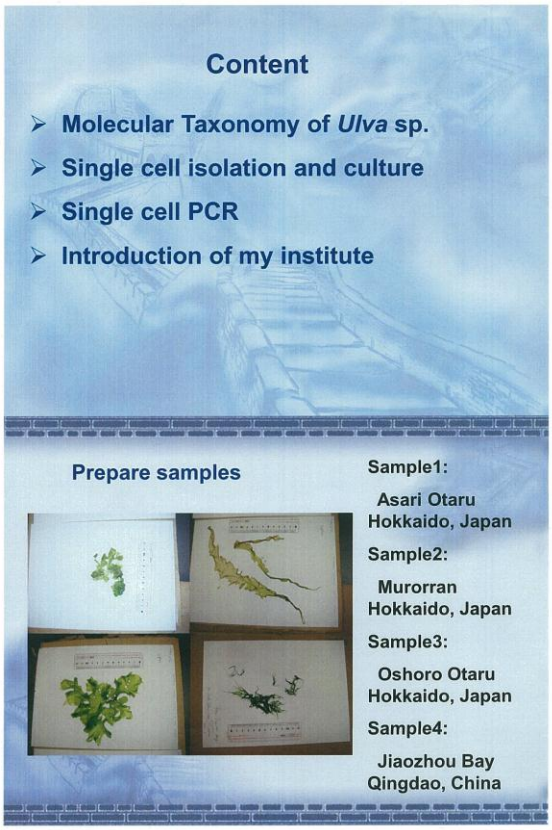
These primers were designed by: Nakayama T., Watanabe S., Mitsui K., Uchida H. & Inouye I. 1996. The phylogenetic relationship between the Chlamydomonadales and Chlorococcales inferred from 18S rDNA sequence data. Phycological Research 44: 47-55.

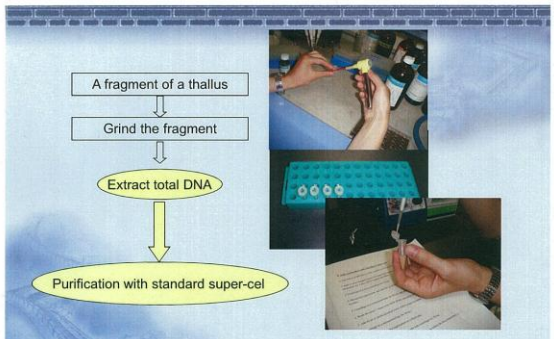
Appendix 7 Power Point Slides of Training Results of Trainees

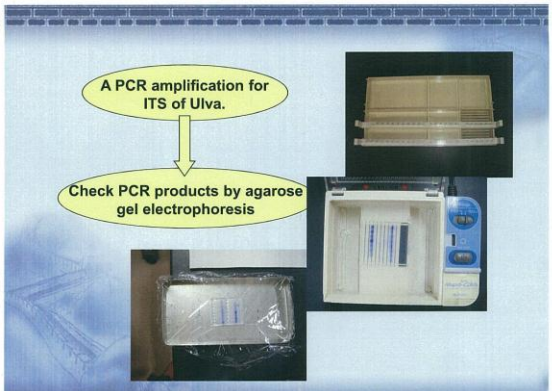
2010

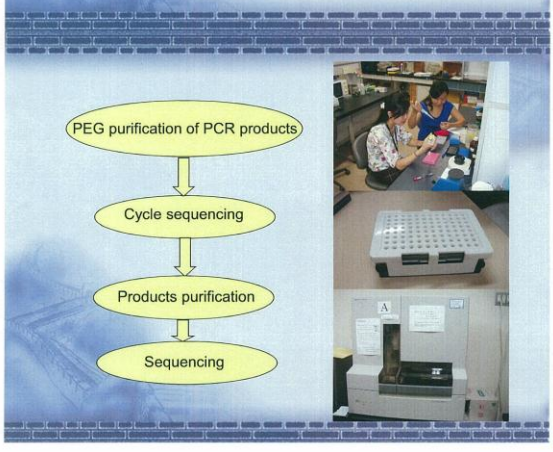
- Han XIAOTIAN (CHINA)

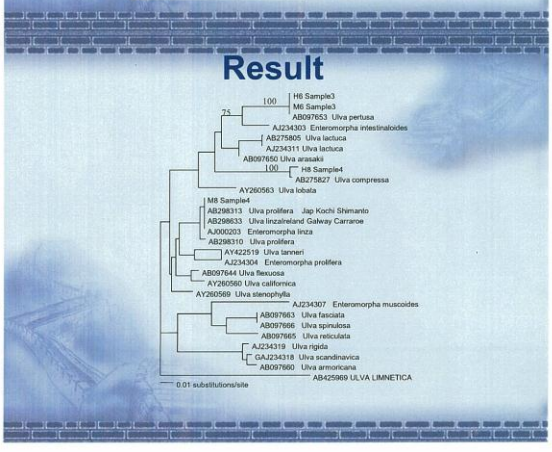




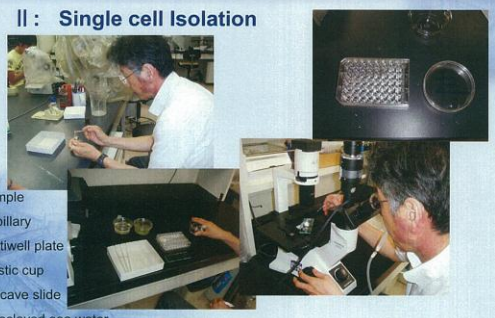








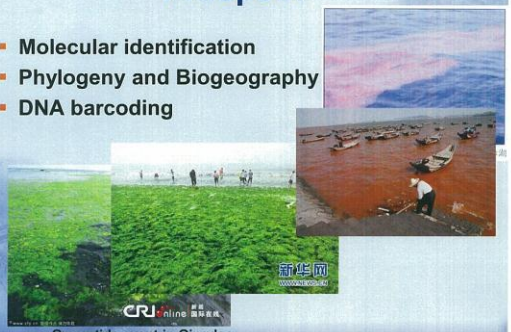
Ⅱ: Single cell Isolation



Sample
Capillary
Multiwell plate
Plastic cup
concave slide
Autoclaved sea water
Silicone tube

Prospect

- Molecular identification
- Phylogeny and Biogeography
- DNA barcoding



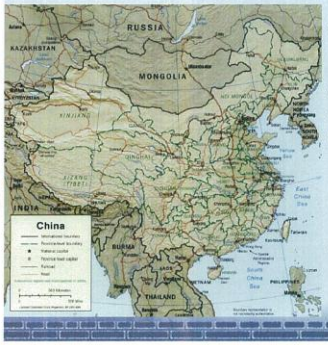
Green tide event in Qingdao-

Ⅲ: Single cell PCR

- One microalgae cell
- One micro glass bead (autoclaved)
- One microlitre sterile distilled water

PCR tube(cell broken by vibration)

Introduction

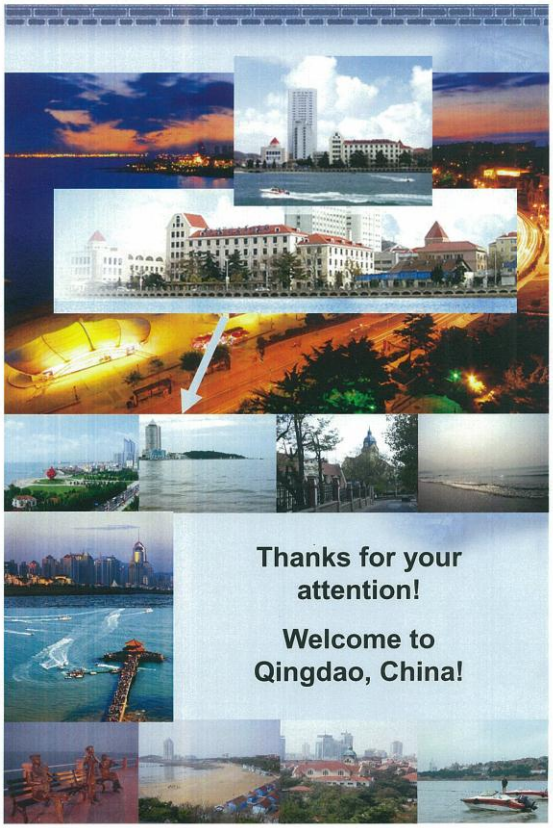


- Location:
  Eastern Asia,
  West Coast of Pacific Ocean
- Coastline: 18,000 Km
- Four sea regions:
  Bohai Sea
  Yellow Sea
  East China Sea
  South China Sea
- Three climatic zones:
  Warm Temperature
  Subtropical
  Tropical

- Location:
north latitude 35。35' to 37。09' and east longitude 119。30' to 121
- Total area:
10,654 square kilometers
- Coastline:
862.64 kilometers
- sea regions:
East and south Yellow Sea
- climatic zones:
Temperate

Research Divisions

Experimental Marine Biological Laboratory, CAS
Key Laboratory of Marine Ecology & Environmental Sciences, CAS
Key Laboratory of Ocean Circulation and Wave Studies, CAS
Key Laboratory of Marine Geology and Environment, CAS
R&D Center of Marine Biotechnology, CAS
R&D Center of Marine Environmental Engineering & Technology
Department of Marine Organism Taxonomy & Phylogeny
Center for Marine Corrosion & Protection



Thanks for your attention!

Welcome to Qingdao, China!

- Minhthanh Thi NGUYEN (VIET NAM)

CAPaBLE Training Program

Molecular Taxonomy of Marine Macroalgae

Hokkaido University
July 5 – 9, 2010

NGUYEN THI MINH THANH
Institute of Biotechnology, Vietnamese Academy of Science and Technology, Viet Nam

Contents

I. Training program in Hokkaido University

II. Achievements

III. Perspective of future research in Viet Nam

I. Training program in Hokkaido University

by Takeo Horiguchi and Kazuhiro Kogame
(The Phycological Laboratory, Faculty of Science, Hokkaido University)

1. Samples for DNA extraction
2. DNA extraction
3. DNA purification with Standard Super-Cel
4. PCR
5. Agarose gel electrophoresis

I. Training program in Hokkaido University

(cont.)

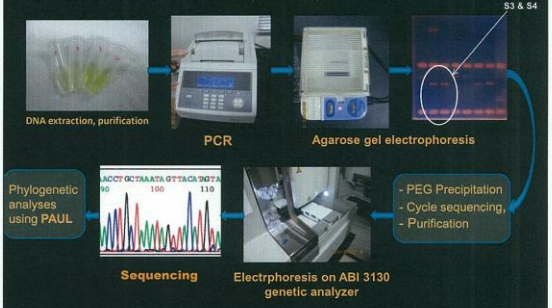
6. PEG precipitate (Purification of PCR products)
7. Cycle sequencing
8. Purifying cycle sequencing products
9. Electrophoresis on ABI 3130 genetic analyzer
10. Aligning DNA sequences
11. Phylogenetic analyses using PAUL

II. Achievements

1. Phylogenetic analysis using *Ulva* species for the ITS region

Samples for DNA extraction (Frozen samples)

1. Phylogenetic analysis using *Ulva* species for the ITS region *(illustrate)*



1. Phylogenetic analysis using *Ulva* species for the ITS region *(illustrate)*

Appendix

* Collection data of samples used in this course

Ulva spp

Oshoro, Otaru, Hokkaido, Japan; 15 June 2010; leg. K. Kogame

* Information of primers used in this course

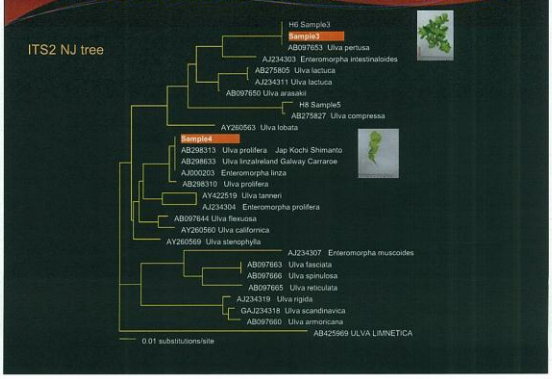
Primers to amplify the ITS1-5.8S-ITS2 region of *Ulva* species

ITS1 (forward): 5'-TTTGTACACACCGCCCG-3'

ITS4 (reverse): 5'-TCCTCCGCTTATTGATATGC-3'

1. Phylogenetic analysis using *Ulva* species for the ITS region

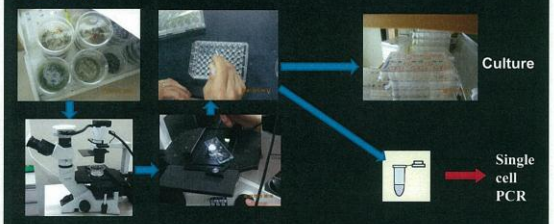
ITS2 NJ tree



II. Achievements

2. Supplements

* Isolate Microalgal Single Cell (*for culture, Single cell PCR, ...*)



II. Achievements

2. Supplements

* Sightseeing

Culture rooms with different temperatures

II. Achievements

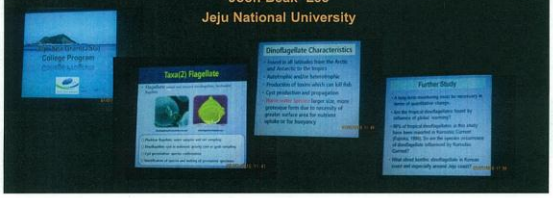
2. Supplements

* Seminar

"Korean Indigenous Species Survey and Occurrence of Tropical Dinoflagellates around Jeju Island"

Joon-Beak Lee

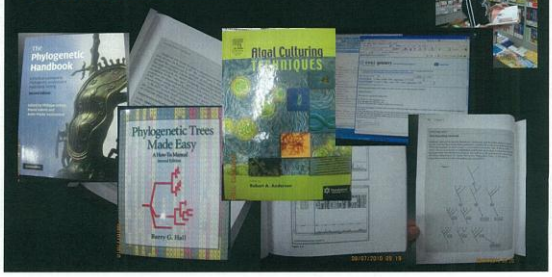
Jeju National University



II. Achievements

2. Supplements

* Collect Documents



Practise

Practise

III. Perspective of future research in Viet Nam

* Seaweed in Vietnam

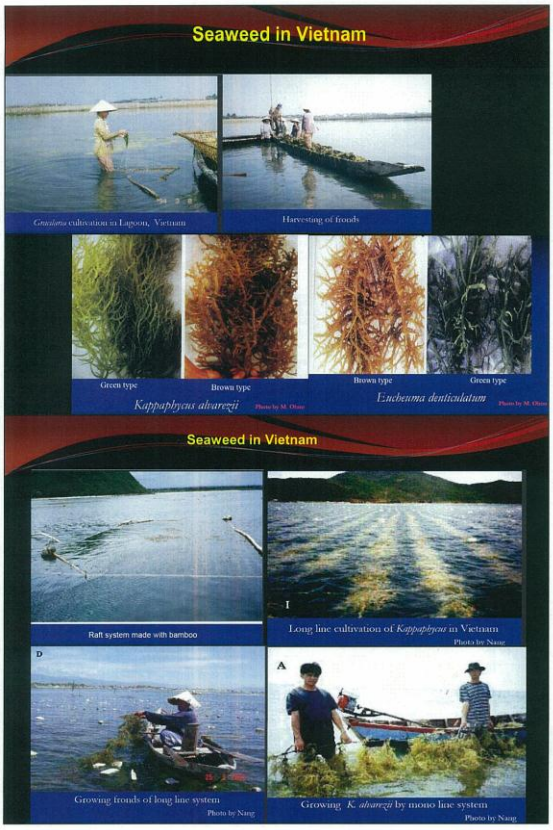
- Vietnam is a tropical country and has coastline approx. 3200 km with diversity of seaweed flora.
- Total seaweed species: approx. 1000

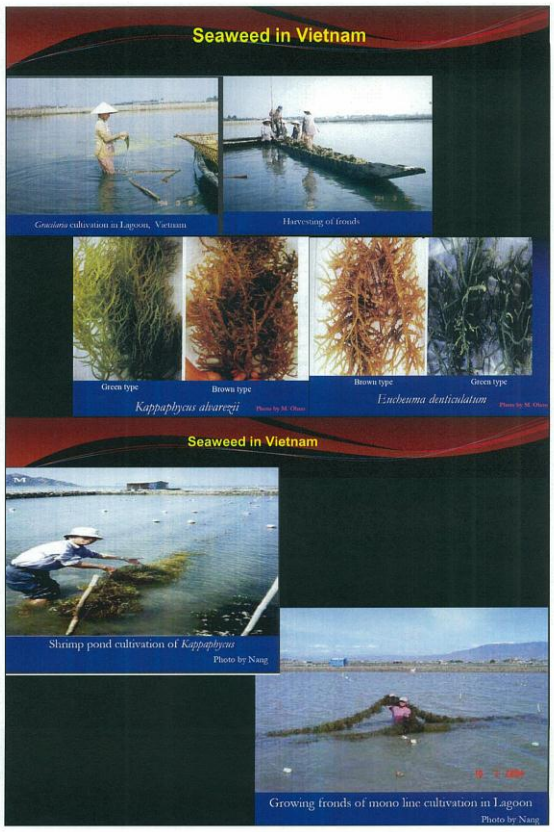
(Pham, 1969;. Huynh and Nguyen, 1998)

- About 638 species have been identified
  - 269 Rhodophyta
  - 143 Phaeophyta
  - 151 Chlorophyta
  - 76 Cyanophyta

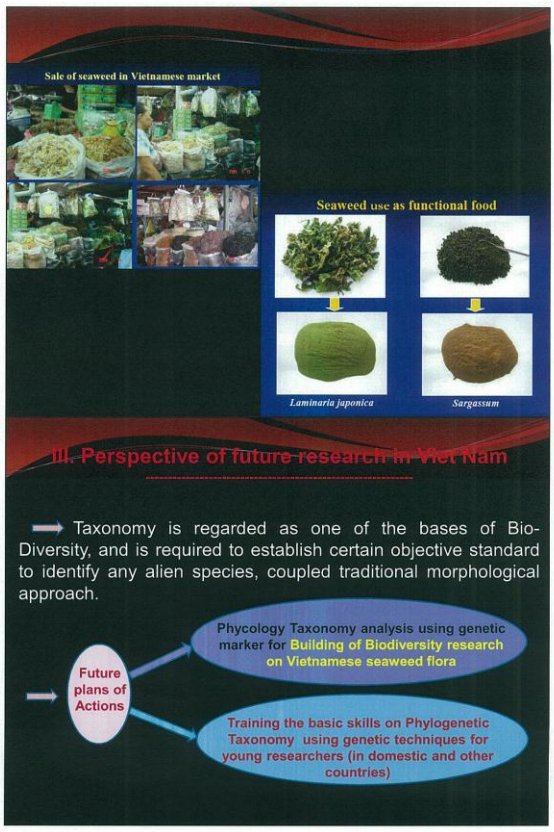
Seaweed in Vietnam

CBA2011-01CMY-Kawai-FINAL REPORT


Seaweed in Vietnam
Gracilaria cultivation in Lagoon, Vietnam
Harvesting of froods
Green type
Brown type
Kappaphycus alvarezii
Brown type
Green type
Eucheuma denticulatum
Seaweed in Vietnam
Raft system made with bamboo
Long line cultivation of Kappaphycus in Vietnam
Photo by Nang
Growing fronds of long line system
Growing K. alvarezii by mono line system


Seaweed in Vietnam
Gracilaria cultivation in Lagoon, Vietnam
Harvesting of froods
Green type
Brown type
Kappaphycus alvarezii
Brown type
Green type
Eucheuma denticulatum
Seaweed in Vietnam
Shrimp pond cultivation of Kappaphycus
Photo by Nang
Growing fronds of mono line cultivation in Lagoon
Photo by Nang


Seaweed in Vietnam
Seaweed
- Seaweed are the potential source of revenue for our country.
- Several Vietnamese seaweed species have economic importance as food for human, as industrial materials, as ingredients in traditional medicine, and as biofertilizers.
Vietnamese seaweed soup
SEAWEED
III. Perspective of future research in Viet Nam
* Curent researchs
- There were many seaweed genus/species studied on nutrition values, physiological activities on animals, … and molecular biology (Ulva, Gracilaria, Kappaphycus, Caulerpa, Hypnea, Sargassum…)
- Molecular Taxonomy has used efficiently for studies on seaweed biodiversity, phylogenetic, identification species… (including usefull and toxic seaweeds: Alexandrium, Prorocentrum, Pseudonitschia, Coolia, Gambierdiscus, Microcystis, Occilatoria....)
Ulva lactuca


Sale of seaweed in Vietnamese market
Seaweed use as functional food
Laminaria japonica
Sargassum
III. Perspective of future research in Viet Nam
Taxonomy is regarded as one of the bases of Bio-Diversity, and is required to establish certain objective standard to identify any alien species, coupled traditional morphological approach.
Future plans of Actions
Phycology Taxonomy analysis using genetic marker for Building of Biodiversity research on Vietnamese seaweed flora
Training the basic skills on Phylogenetic Taxonomy using genetic techniques for young researchers (in domestic and other countries)

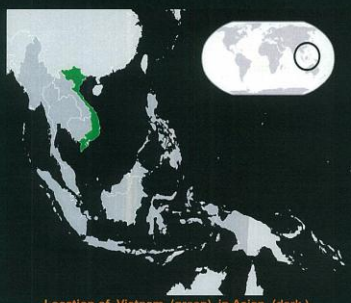
III. Perspective of future research in Viet Nam

Taxonomy is regarded as one of the bases of Bio-Diversity, and is required to establish certain objective standard to identify any alien species, coupled traditional morphological approach.

Future plans of Actions

- Participate in the Asian Pacific networks and others to exchange the biodiversity information and propose actions
- Evaluate the seaweed biodiversity using molecular techniques → direct introduced taxa in the early stage

Welcome to Vietnam



Location of Vietnam (green) in Asian (dark)

We always want to collaborate for researchs, actions, training...

→ *Please contact:*

Department of Algal Biotechnology,
Institute of Biotechnology (IBT),
Vietnamese Academy of Science and Technology (VAST), Viet Nam

18 Hoang Quoc Viet - Cau Giay - Hanoi - Vietnam
Tel: + 84 47 9 11059; Fax: + 84 48 363 144
Mobile: 091 534 3660; E-mail: ddhong60vn@yahoo.com

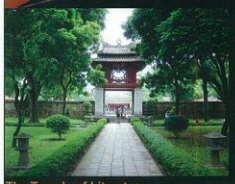
Welcome to Vietnam

Hanoi - The Capital of Vietnam, in North of Vietnam

Turtle Tower in Hoan Kiem Lake, in centeral Hanoi

Highway

Welcome to Vietnam



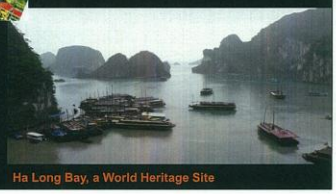
The Temple of Literature Quoc Tu Giam, Hanoi

Presidental Palace, Hanoi

Ho Chi Minh Mausoleum in Hanoi

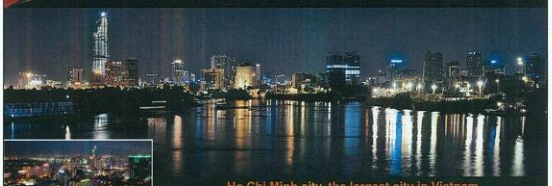
Welcome to Vietnam

Nha Trang city

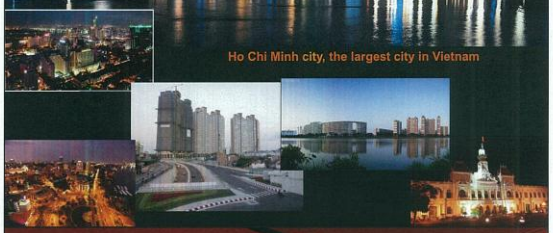


Ha Long Bay, a World Heritage Site

Welcome to Vietnam



Ho Chi Minh city, the largest city in Vietnam



Thank you for listening !

- Anchana PRATHEP (THAILAND)

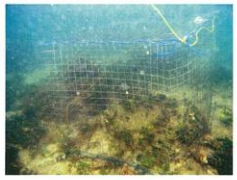
SEAWEED & SEAGRASS Research Unit

CAPaBLE Training Program

Capacity Building of Biodiversity Research in Coastal Zones of the Asia Pacific Region

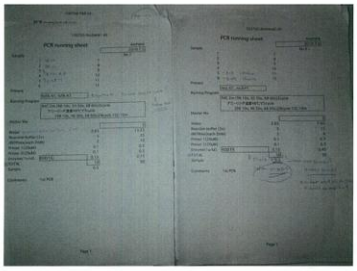
Macroalgal Taxonomy Using Genetic Markers

Anchana Prathep,
Prince of Songkla University, HatYai, Thailand

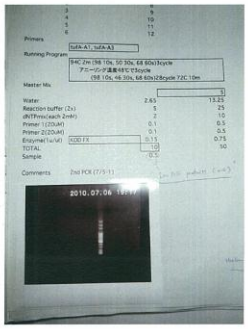


Halimeda systematic, population ecology, resource allocation

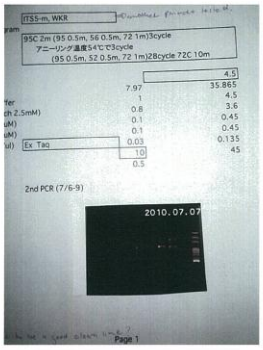
1st Day at the molecular work-WORLD



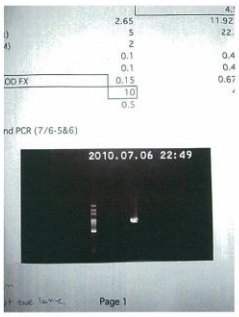
No Bands-what so ever!



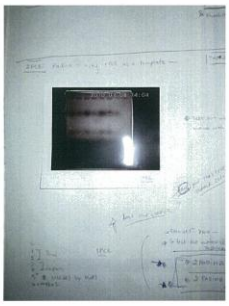
Halimeda world of extraction!



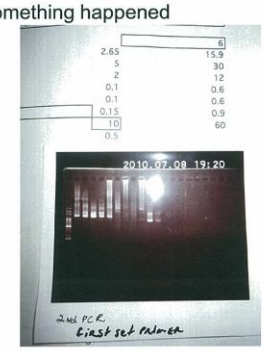
Come to the rescue

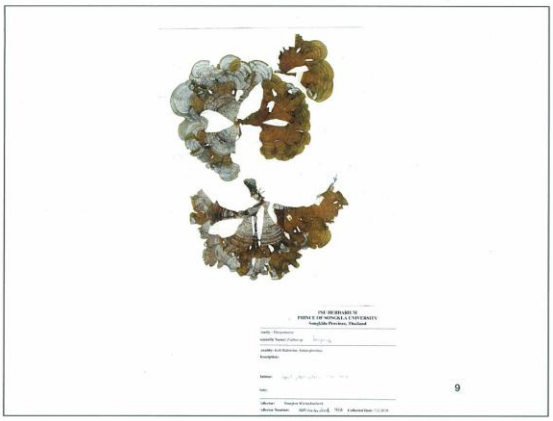


Come to the rescue & what is so wrong with me???

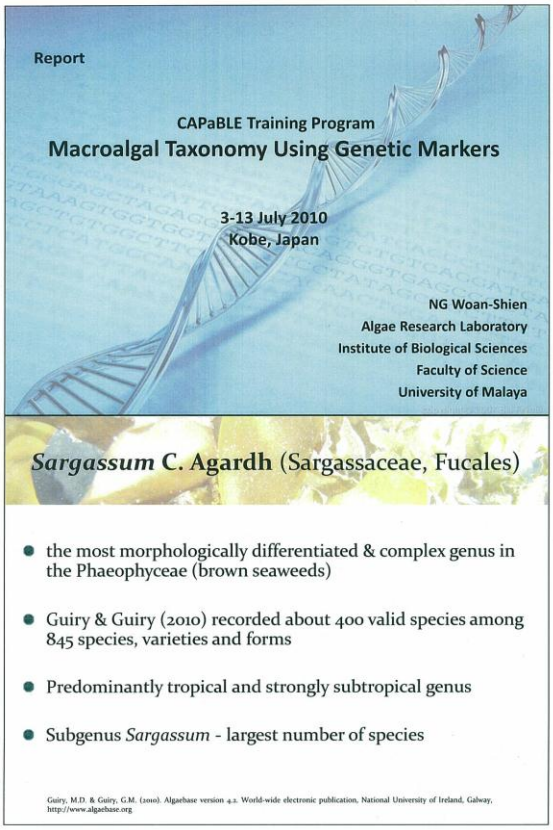


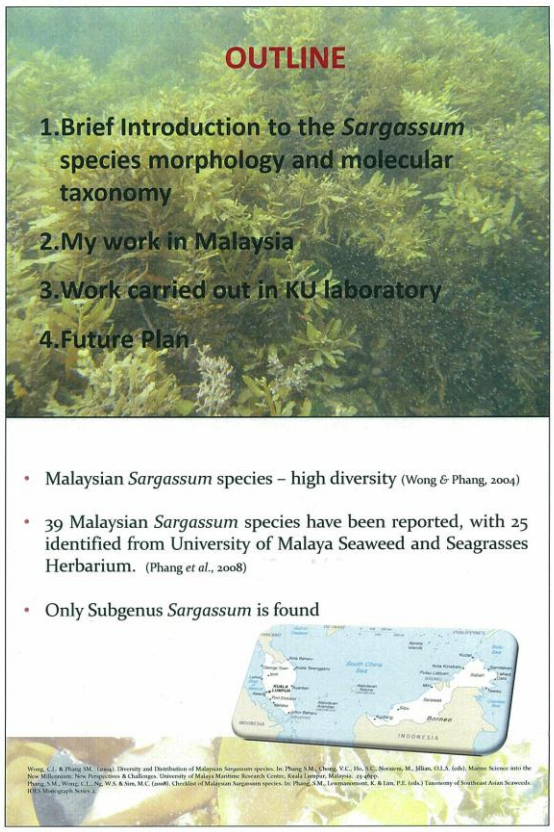
Finally something happened





- Woan-Shien NG (MALAYSIA)





Morphology of *Sargassum*

- Holdfast
- Stem
- Branch
- Laterals/Leaves
- Vesicles
- Receptacles
- Cryptostomata

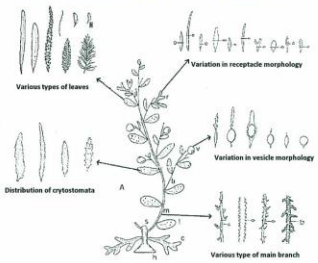


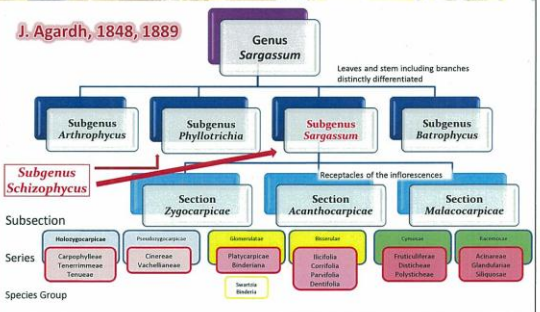
Diagram of a *Sargassum* whole plant.

(b = secondary branch; c = cauline leaf; h= holdfast; l =leaf; m = main branch; r = receptacle; s = stem; v = vesicle). Adapted from Yoshida 1983, 1988.

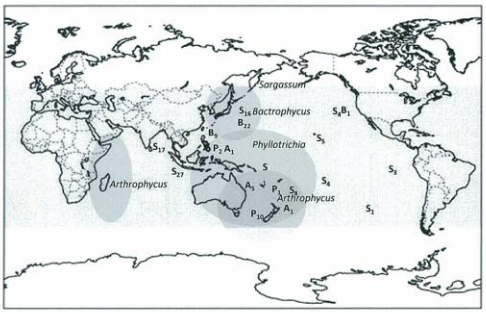
Taxonomy of *Sargassum*

The attribution of a species to a subgenus is not always easy to decide

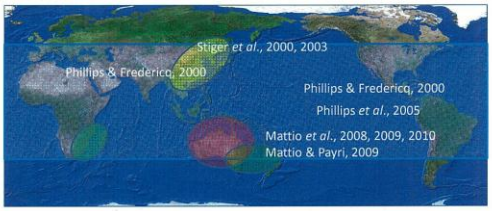
J. Agardh, 1848, 1889



Map of geographical distribution ranges for the various subgenera of genus *Sargassum*



• Phylogenetic Studies on *Sargassum* in the world



Phillips & Fredericq, 2000; Phillips *et al.*, 2005
Stiger *et al.*, 2000, 2003
Mattio *et al.*, 2008, 2009, 2010; Mattio & Payri, 2009

– rbcLS operon
– ITS-2 spacer
– ITS-2 spacer, partial rbcL and spacer, cox3

Objectives of my study:

1. To determine the importance of the morphological characteristics in correlation with phylogenetic analysis.
2. To produce a more comprehensive phylogenetic analysis on the Malaysian *Sargassum* species using sequences of RuBisCo gene (partial *rbc*L and spacer), mitochondrial gene (*cox*3) and Internal Transcribed Spacer-2 region (ITS-2).

Phylogenetic analysis

- A total of 86 partial *rbc*L, 154 ITS-2 region, and 85 *cox*3 sequences, including 28, 40, 26 GenBank sequences respectively, were aligned.
- Total lengths were 1202 bp, 664 bp, and 571 bp respectively for each region, including gaps.
- All taxa identified in this study belong to subgenera *Sargassum* subsection *Zygocarpicae*, *Malacocarpicae* and *Acantocarpicae*.

Molecular Taxonomy

- Some implications:
  - Groupings based on genetic analysis do not always conform with those based on receptacle morphology
  - test the basis of J Agardh's scheme
  - New classification was introduced
  - Finding the best genetic marker(s) is a big challenge … finding the best morphological character(s) to revise the taxonomic approach

OBJECTIVES

By participating in this training program,

To help accomplishing the projects on *Sargassum* and other brown algal systematics through molecular taxonomy

To standardize materials, methods and terms used in the molecular researches.

To support interdisciplinary research and several on-going researches benefit from the active collaboration between Algae Research Laboratory, IOES and other research institutions.

MATERIALS AND METHODS

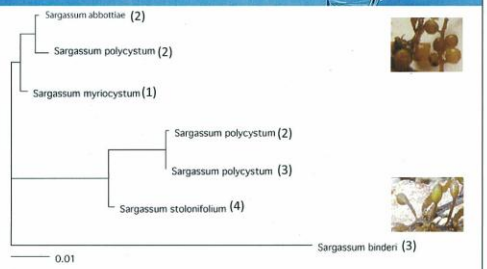
Total samples brought to Kobe, Japan
1.DNA samples (15)
2.Silica-gel dried samples (7)

Total of successfully PCR amplified samples (18) using molecular markers as following:
1.nad3-P1
2.mt16S-P1
3.rps14-sm1

Step-down PCR was performed

Successful PCR products were subjected to PEG purification and cycle sequencing as shown in the manual

Result: ML Tree



- Total lengths were 1467 bp for the region, including gaps
- All taxa in this experiment belong to subgenera *Sargassum* subsection *Zygocarpicae*, *Malacocarpicae* and *Acanthocarpicae*.

Discussions

- Design new primers specifically for samples from this region
- More samples are required for better resolution of the phylogenetic tree
- Possibility of new nomenclature revision of this section

FUTURE PLAN

to have further collaborations for molecular studies such as DNA sequencing data and publications

to join the institute in the University of Malaya after graduation as lecturer, research fellow or post-doc

to pass on the knowledge to the new undergraduate and postgraduate students as well as build up the next generation of seaweed taxonomists for the university and country

ACKNOWLEDGEMENTS

Sponsors:
APN Center
International EMECS Center

Thank you !!

Prof. Hiroshi Kawai
Dr. T. Hanyuda
Dr. A. Kurihara
And lab members of KU labo

THE END ☺

- Soradkorn PHIMLA (THAILAND)

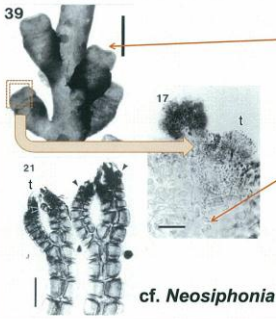
Macroalgal Taxonomy Using Genetic Markers
: A case study on Genus *Laurencia* complex

Soradakorn Phimla

Kasetsart University

CAPaBLE Training Program
Capacity Building Biodiversity Research in Coastal Zones of the Asia Pacific Region
3-13 July 2010
Kobe university, Fukui Prefecture University and Hokkaido University

The genus *Laurencia* (sensu lato) can be distinguished from the other Rhodomelacean genera by:



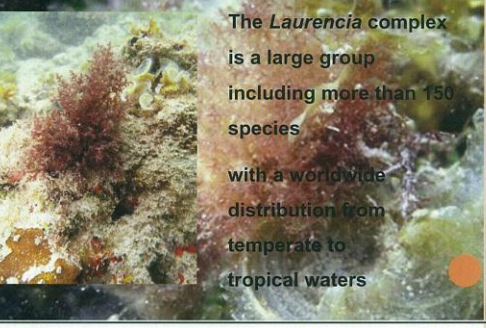
1) apical cells are sunk in apical pits of blunt branchlets

2) a central axis is recognizable only near the apical cell

3) an extensive cortex is formed

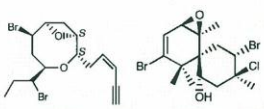
cf. *Neosiphonia* t: trichoblast

Genus *Laurencia* complex



The *Laurencia* complex is a large group including more than 150 species with a worldwide distribution from temperate to tropical waters

Genus *Laurencia* complex



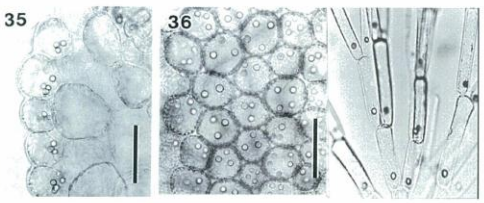
laureatin
(C15 acetogenin)

prepacifenol
(sesquiterpen)

The first report of such halogenated compounds was from the sea hare *Aplysia*. They consume *Laurencia and store those* compounds as "chemical weapons".

Genus *Laurencia* complex

corps en cerise (=cherry bodies)



Oil-body like cell inclusions.
Storage of halogenated secondary metabolites.

Genus *Laurencia* complex

The genus *Laurencia* complex ⇐ Lamouroux 1813

- The genus *Laurencia*
- The genus *Osmundea* — Nam *et al.* 1994
- The genus *Chondrophycus* — Garbary & Harper 1998; Nam 1999
- The genus *Palisada* — Nam 2006; Nam 2007
- The genus *Yuzurua* — Martin-Lescane *et al.* 2010

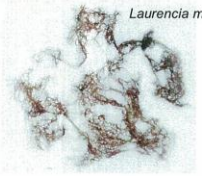
Genus *Laurencia* complex

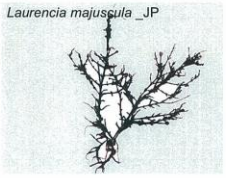
Figure 3.3.7. Antibacterial activity of halogenated compounds isolated from the Japanese *Laurencia* spp. against marine bacteria.

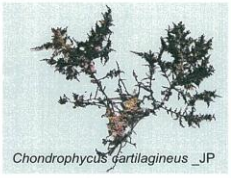
Source of functional Pharmacological compounds (antibiotic substances etc.)

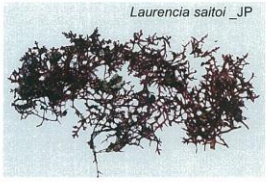
Genus	Vegetative periaxial cell	2ry pit connection	*Corp en cerise*	Spermatangial initial
Osmundea	2	+	-	Filament type
Laurencia	4	+	+	Tricoblast type
Chondrophycus	2	-	-	Tricoblast type
Palisada	2	-	-	Tricoblast type
Yuzurua	2	+	-	Tricoblast type

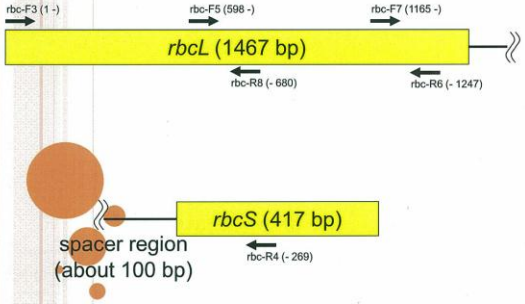
Extraction, amplification and sequencing











How to design primer

Primers used for amplification and sequence in this study

rbc-F3 : ATGTCTAACTCTGTAGAAGAACGGAC

rbc-R4 : CCTCTWGTATTATCAAAWGCRTT
(reverse-complement: AAYGCWTTTGATAATACWAGAGG)

rbc-F5 : GAAGGYYTWAGAGGTGGWYTAGA

rbc-R6 : CCAATWGTACCWCCWCCRAAYTG
(reverse-complement: CARTTYGGWGGWGGTACWATTGG)

rbc-F7 : GGTATYCAYTGYGGWCARATGC

rbc-R8 : ATCTYTCTTTCCARCGCATRAAWGG
(reverse-complement: CCWTTYATGCGYTGGAAAGARAGAT)

DNA EXTRACTION

- using the Sigma GenElute™ Plant Genomic DNA miniprep Kit
- Modified Chelex extraction of DNA for PCR (by Zuccarello)

•Grinding plant tissue with plastic pestle in microfuge tube

PCR amplification

process	Tempature (°C)	time	cycle
Denature	94	2 min	
Annealing	50	30 sec	30 cycles
Extension	68	1 min	

PCR product → **Check PCR product**
(Agarose-gel eletrophoresis)

Cycle Sequencing

process	Tempature (°C)	time	cycle
Rapid thermal ramp	94	2 min	30 cycles
	50	30 sec	
	68	1 min	

PCR template → Purification PCR Product

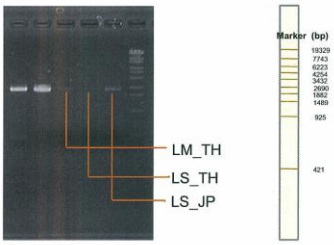
(EXOSAP)

↓

Sequencing

rbc-F3

ATGTCTAACTCTGTAGAAGAACGGAC



rbc-R4

CCTCTWGTATTATCAAAWGCRTT

(reverse-complement : AAYGCWTTTGATAATACWAGAGG)

Sequence alignment and phylogenetic analyses

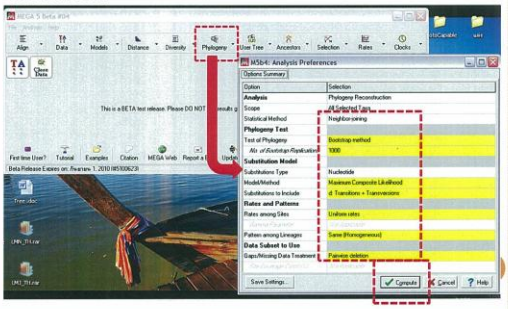
sequences from (GeneBank + specimen) was confirmed and edited by Bioedit

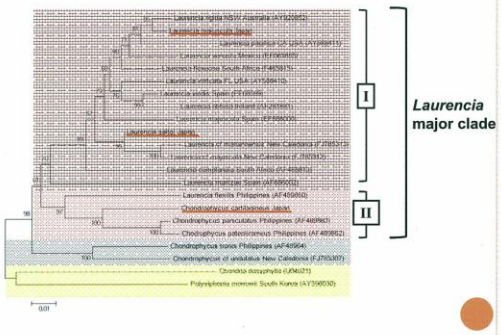
OUT GROUP

- *Polysiphonia morrowii* SouthKorea (AY396030)
- *Chondria dasyphylla* (U04021)
- *Chondrophycus cf. undulatus* New Caledonia(FJ785307)
- *Chondrophycus tronoi* Philippines (AF48964)
- *Chondrophycus paniculatus* Philippines (AF489863)
- *Chodrophycus patentirameus* Philippines (AF489862)

Phylogenetic analyses of rbcL

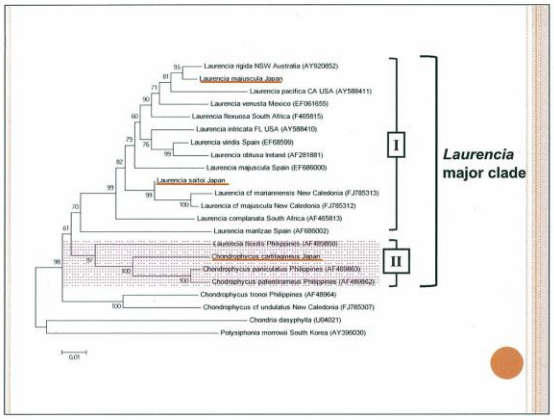
MEGA 5 Beta





L. majuscula Thailand

L. majuscula Japan



THANK YOU FOR YOUR ATTENTION

- Roike Iwan MONTOLALU (INDONESIA)

Macroalgal Taxonomy Using Molecular Markers : In case study *Kappaphycus*

July 12, 2010

CAPaBLE Training Program
Capacity Building Biodiversity Research in Coastal Zones of the Asia Pacific Region

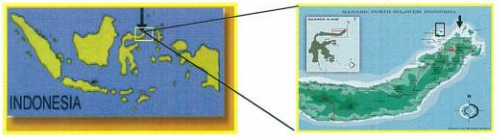
Kobe University, Fukui Prefecture University and Hokkaido University

Roike Iwan Montolalu

Introduction

- The seaweeds most commonly cultivated for the carrageenan industry belong to the genera *Kappaphycus* and *Eucheuma*
- The formal taxonomy of these taxa has for a long time been in confusion due to misapplication of commercial and scientific names, the known general paucity of adequate morphological characters and the morphological plasticity of seaweeds.
- Much of the taxonomic confusion was addresed by the pioneering work of Maxwell Doty (Doty, 1985, 1988; Doty & Norris, 1985)
- Even in detailed work of Doty, variability in the presence or absence of diagnostic morphological characters within taxa was noted, especially in non-ideal specimens (non-reproductive specimens and specimens lacking typical attachement structures).

Collection samples



Kappaphycus alvarezii (Doty) Doty ex P.C. Silva (Gigartinales, Solieriaceae) was collected by hand on the coast of Likupang North Sulawesi, Indonesia, which is in a tropical zone. Immediately after sampling, the seaweed was washed several times with clean seawater in order to remove nonalgal materials. (June 28, 2010)

Samples were placed in silica gel until DNA extraction.

Out Group

- *Gracilaria vermiculophylla* (Ohmi)

[Tateyama Banda, March 24, 2008]

http://www.research.kobe-u.ac.jp/rcis-kurcis/KURCIS/KaisoHyohonHP/Osaka-bay/Gracilaria%20ver-O.html

DNA EXTRACTION

•Grinding plant tissue with plastic pestle in microfuge tube

- using the Sigma GenElute™ Plant Genomic DNA miniprep Kit
- PCR Amplification
- •Check of PCR (Agorose-gel elektrophoresis)
- •Purifiication of PCR Products
- •Sequencing
- •Confirmation and edit of sequencing (BioEdit)
- •Phylogenetic Analysis

Primers used for amplification and sequence in this experiment

rbc-F3 : ATGTCTAACTCTGTAGAAGAACGGAC

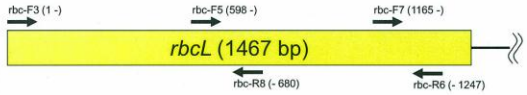
rbc-R4 : CCTCTWGTATTATCAAAWGCRTT
(reverse-complement: AAYGCWTTTGATAATACWAGAGG)

rbc-F5 : GAAGGYYTWAGAGGTGGWYTAGA

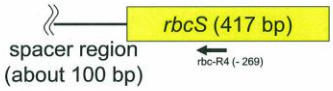
rbc-R6 : CCAATWGTACCWCCWCCRAAYTG
(reverse-complement: CARTTYGGWGGWGGTACWATTGG)

rbc-F7 : GGTATYCAYTGYGGWCARATGC

rbc-R8 : ATCTYTCTTTCCARCGCATRAAWGG
(reverse-complement: CCWTTYATGCGYTGGAAAGARAGAT)

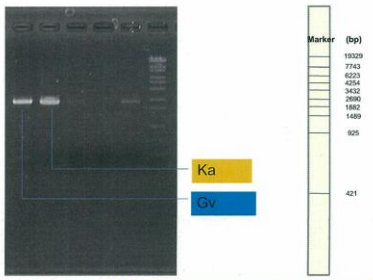


Rbc-F3 and rbc-R4 was used PCR
Several primers used for each samples



rbc-F3

ATGTCTAACTCTGTAGAAGAACGGAC



Check of PCR (Agorose-gel elektrophoresis)

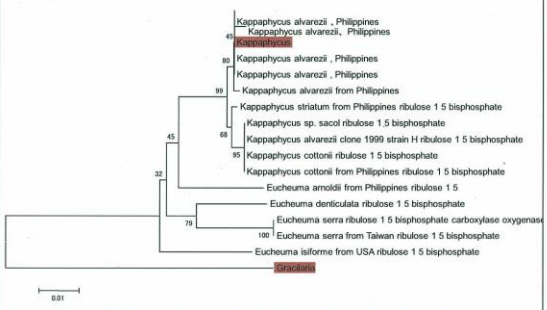
Amplification

Polymerase chain reaction

process	Tempature (°C)	time	cycle
Denature	94	2 min	
Annealing	50	10 sec	
	(Tm-5)	30 sec	30 cycle
Extension	68	1 min	

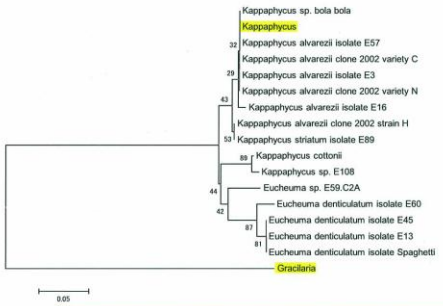
PCR product → Check PCR product

rbcL



Phylogenetic tree of *Kappaphycus alvarezii* from rbcl

Rubisco spacer



Phylogenetic tree of *Kappaphycus alvarezii* from rubisco spacer

2011

- Tsz Yan NG (CHINA)

APN

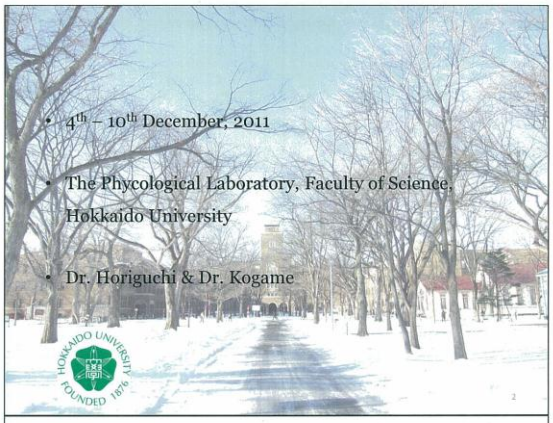
CAPaBLE Training Program

on

Molecular Taxonomy of Marine Macroalgae

NG Tsz Yan
School of Life Sciences
The Chinese University of Hong Kong

1



- 4th – 10th December, 2011
- The Phycological Laboratory, Faculty of Science, Hokkaido University
- Dr. Horiguchi & Dr. Kogame

2

Schedule

Dec 5	DNA extraction, DNA purification
Dec 6	PCR, Electrophoresis
Dec 7	PEG Precipitation, Cycle Sequencing
Dec 8	Purification, Sequencing
Dec 9	Phylogenetic analysis

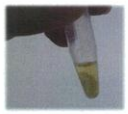
3

DNA Extraction

- For algae and plants DNA extraction
  - CTAB
  - DNeasy Plant Mini Kit
- Method modified by the laboratory
  - ✓ Simple
  - ✓ Low-cost

4

DNA Extraction



Fragment of a thallus

- YK Buffer
- 2-mercaptoethanol
- HTE Buffer
- Urea
- Chloroform
- Sodium acetate
- Isopropanol
- 70% ethanol

5

DNA Purification

- To obtain clean DNA as template for PCR

DNA solution

NaI

Standard Super-Cel suspension

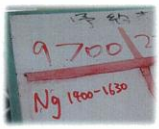
Washing with NEW Wash

Cleaned DNA

6

Polymerase Chain Reaction

- Reaction mixture
  - Taq
  - Buffer
  - dNTP Mixture
  - Forward primer
  - Reverse primer
  - Template DNA
  - Sterilized distilled water
  - DMSO



7

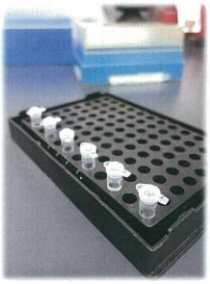
Agarose Gel Electrophoresis

- Agarose + TAE buffer

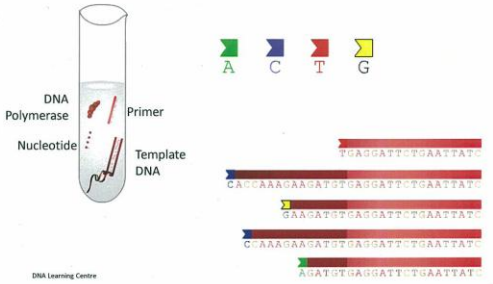
8

PEG Precipitation

- To purify PCR products
- PEG-NaCl solution
- Ethanol
- Distilled water



Cycle Sequencing

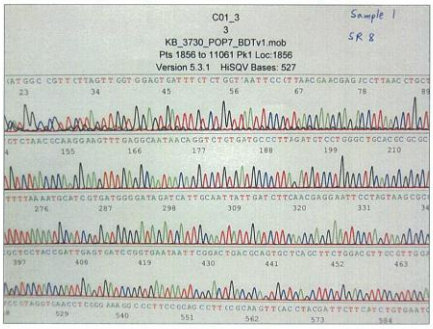


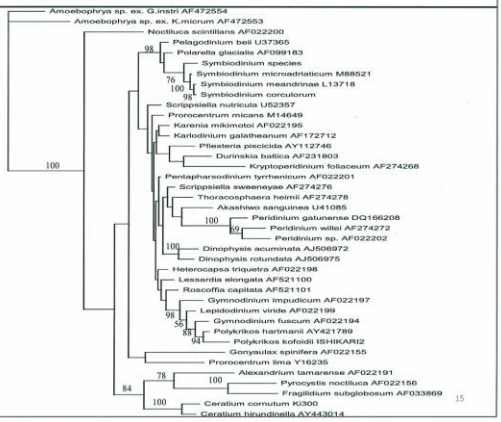
Cycle Sequencing Products Purification

- Ethanol
- Sodium acetate
- EDTA

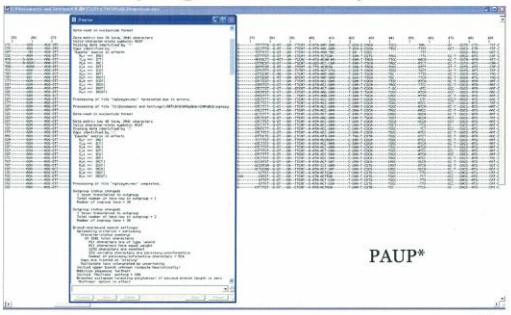
Sequencing

Phylogenetic analysis





Phylogenetic analysis



PAUP*

Acknowledgements

APN

International EMECS Center

- Chea PHALA (CAMBODIA)

Phycology Taxonomy Analysis Training Using Genetic Marker
(APN CAPaBLE Program), 04 – 12 December, 2011
Fukui Prefecture University, Obama, Japan

Taxonomy of Marine Macroalgae Using Molecular Markers

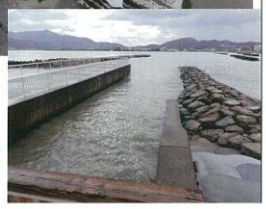
Chea Phala
Marine Aquaculture Research and Development Center
Fisheries Administration, Cambodia
Mobil: (+855) 17 328 345 or (+855) 99 753 783
E-mail: cheaphala76@yahoo.com

Contents of Presentation

- Species and sampling site
- Steps of protocol
- Data analysis
- Results and discussion
- Achievement from training
- Perspectives of future research program

1. Species and sampling site

Ulva species



Ningyo – Hama, Obama, Japan

2. Steps of protocol

1. DNA extraction
2. PCR amplification
3. Check of PCR products
4. Purification of PCR products
5. Cycle sequencing
6. Sequencing

DNA extraction

Two different methods:

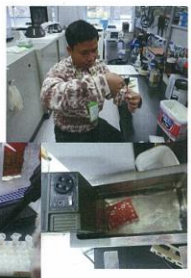
1. Modified Chelex extraction (Simple method)
2. GenElute Plant Genomic DNA Miniprep Kit

Simple method:

- Grind seaweed tissue
- Boil (95 – 100 °C) for 10 mn
- Cool on ice 5 mn and spin 10 mn
- Remove supernatant to new tube
- Use directly for PCR

GenElute Plant Genomic DNA Miniprep Kit

1. Prepare Sea weed tissue 23g (Grind in lysis solution)
2. Release DNA (Lyse, precipitate, and spin)
3. Filter Lysate (Spin 1 minute)
4. Prepare column (Add solution and spin)
5. Bind DNA (Bind and spin twice)
6. Wash column (Wash and spin twice)
7. Elute DNA (Elute and spin twice)



PCR amplication

Prepare DNA with total volume 40µl

Setting of PCA program

Begin PCR by thermal cycler

•Distilled water	8.0 µl
•2xPCR Buffer	20 µl
•dNTPs (2.0mM)	8.0 µl
• F. Primer (20µM)	0.8 µl
•R. Primer (20µM)	0.8 µl
•Polymerase	0.4 µl
•DNA Template	2.0 µl

Check of PCR products

Prepare Agarose-gel (Agarose + TAE buffer)

Cast a gel (Supplied tray + Comb)

Load PCR 1µl loading buffer +5µl PCR

Run electrophosis (25 minute)

UV light and photograph

Purification of PCR products

PEG solution (Incubate on ice 1h and spin 15 mn)

Remove PEG supernatant

Wash pellet 2 times (Ethanol and spin 10 mn)

Dry the pallet 10 mn and Re-suspend DNA in 50µl DWater

Cycle seqencing

Prepare of DNA sequencing reaction By Bigdye kit with total volume 20 µl

-Distilled water 13.5 µl
-DNA Template 1 µl
-5x buffer 4.0 µl
-Primer (10 µl) 1 µl
-Big dye 0.5 µl

Thermal cycling program
96 °C : 1mn/ 95 °C : 10Sec.,
50 °C : 5sec., 60 °C : 4mn

*Bigdye product purification
-5µl EDTA + 60µl ethanol, spin 30 mn and remove liquid
- 60µl ethanol, spin 10 mn and remove liquid
- Dry 10 mn in room temperature
- 15µl HiDi keep single band
- Heat 100 °C for 5 mn and ice 5 mn

Cycle sequencing

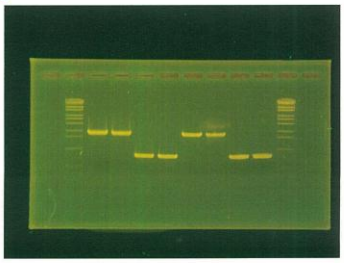
3. Data analysis

➢ Construct molecular phylogenetic trees

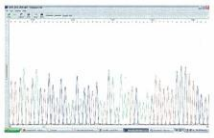
- Chromas Lite Software was used for ABI electropherogram view, editing, and Reverse Complement if it was complementary sequence
- Download any sequence data from National Center for Biotechnology Infromaton (NCBI) website
- Using BioEdit Sequence Alignment Editor Software for data alignment and sequence
- Phylogenetic Trees by using MEGA5 Software

4. Results and discussion

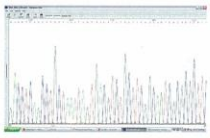
PCR products



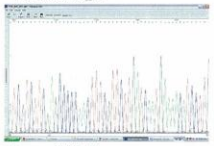
Result of cycle sequencing



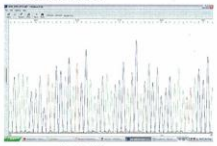
Premer CL18_5F



Premer CL18_7F

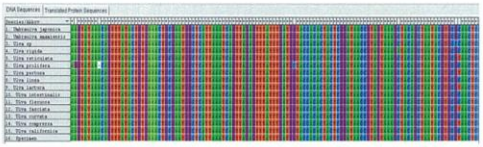


Premer CL18_4R

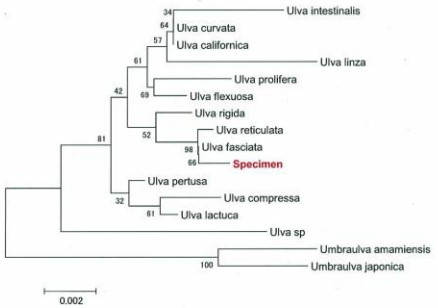


Premer CL18_6R

Data alignment and sequence



Result of 18s analysis
Phylogenetic Tree based on rbcL gene



Discussion

- According to result of sequencing and Phylogenetic Tree based on rbcL gene the identification of Ulva species are clarified, specimen is close to *U. faciata* and *U. reticulata*, but *U. reticulata* is not much appear in Japan sea.
- Ulva species was collected in Ningyo Hama, Obama on 04 December 2011 is more closely to *U. fasciata* species
- So the research was carried out successfully

Ulva fasciata in Ningyo Hama, Obama, 04 December 2011

6. Perspectives of future research program

- The knowledge I obtained is important for me and especially for my center, to make plan and setup research program by using above technology for Cambodia.
- To set up laboratories with full facilities and equipments for this research in Cambodia
- To collaborate with countries network to conduct research and exchange information are related to phycology taxonomy analysis
- To transfer and pass on this technology to the new staffs, students, as well as to build up the next generation of seaweed taxonomists in center, institute, and university in Cambodia

5. Achievement from training

- I obtained from training include technical and skills such as: DNA extraction, PCR amplification, Check of PCR products, Purification of PCR products, Cycle sequencing, sequencing, and especially how to dry the sample and data analysis.
- This training was very important for me to get new technology of macro-algae taxonomy by using molecular makers.
- To give me opportunity to create strong networking amongst participants and countries.

Acknowledgement

- I would like to express special thank to International Center for Environment Management of Enclosed Coastal Seas and Asia-Pacific Network for Global Change Research for their supporting this training.
- I would like to express my gratitude and high appreciation to instructors/ professors, especially to Dr. Mitsunobu KAMIYA for his instructing and useful comment and helpful during the training .
- I would highly appreciate to organizers for smoothly arrangement and successful training, especially to Ms. Ayako Yamada for her helpful and useful information.
- Many thanks also due to ungraduated students for their kind assistance during the training in Fukui Prefecture University.

- Thi Dinh NGOCMAI (VIET NAM)

APPLICATION OF MOLECULAR MARKER TECHNIQUES IN TAXONOMIC IDENTIFICATION OF A *ULVA* SPECIES COLLECTED FROM OBAMA BAY

Trainee: Ngocmai Thi Dinh (Vietnam)
Supervisor: Prof. Mitsunobu Kamiya (Fukui Prefectural University)

December 11, 2011 CAPaBLE

ULVA SPECIES

- *Ulva* is reportedly good source of protein and vitamins and is usually used in Japan and other East Asian countries for human consumption
- Their identification is of primary importance

MATERIALS AND METHODS

- Material: One species of *Ulva* was collected from Obama Bay (Japan)



MATERIALS AND METHODS–DNA EXTRACTION

1. GenElute Plant Genomic DNA Miniprep Kit

- Grind 14 mg of tissue
- Add 350 mL of Lysis Solution A and 50 mL of Lysis SolutionB to the tube; thoroughly mix, then incubate the mixture at 65 ° C for 10 minutes.
- Add 130 mL of Precipitation Solution to the mixture; mix completely, place the sample on ice for 5 minutes, centrifuge the sample at 14,000 rpm for 5 min.
- Carefully pipette the supernatant from step 3 onto a GenElute filtration column. Centrifuge at 14,000 rpm for 1 minute, discard the filtration column, but retain the collection tube.
- Add 700 mL of Binding Solution directly to the flow-through liquid from step 4, mix thoroughly
- Insert a GenElute Miniprep Binding Column into a microcentrifuge tube. Add 500 mL of the Column Preparation Solution to each miniprep column and centrifuge at 12,000 rpm for 1 minute. Discard the flow-through liquid.
- Carefully pipette 700 mL of the mixture from step 5 onto the column prepared in step 6 and centrifuge at 14,000 rpm for 1 minute. Discard the flow-through liquid; retain the collection tube. Return the column to the collection tube. Apply the remaining lysate from step 5 onto the column. Repeat the centrifugation as above and discard the flow-through liquid and collection tube.
- Place the binding column into a fresh 2 mL collection tube and apply 500 mL of the diluted Wash Solution to the column. Centrifuge at 14,000 rpm for 1 minute. Discard the flow-through liquid, but retain the collection tube.
- Apply another 500 mL of diluted Wash Solution to the column and centrifuge at 14,000 rpm for 3 minutes to dry the column.
- Transfer the binding column to a fresh 2 mL collection tube. Apply 100 mL of pre-warmed (65 ° C) Elution Solution to the column and centrifuge at 14,000 rpm for 1 minute.

MATERIALS AND METHODS–DNA EXTRACTION

2. Modified Chelex extraction of DNA

- Grind 10 mg of tissue
- Add 200 µl of Chelex solution on the tube contained sample
- Grind with clean microfuge tube grinder
- Boil for 10 minutes
- Cool on ice for 5 minutes. Centrifuge at 14,000 rpm for 10 minutes. Remove supernatant and add to new clean microfuge tube


PRIMER

1. ITS

UITS-2F – 5' –TTCGGGAATTTCGTTGAACCCTC–3'

CLITS–7R – 5' –TCCCTTTTCGCTCGCCGTTACTA–3'

2. 18S rDNA

CL18–1F – 5' –TACCTGGTTGATCCTGCCAG–3'

CL18–2R – 5' –CCCTCCGCAGGTTCACCTAC–3'

MATERIALS AND METHOS–PCR

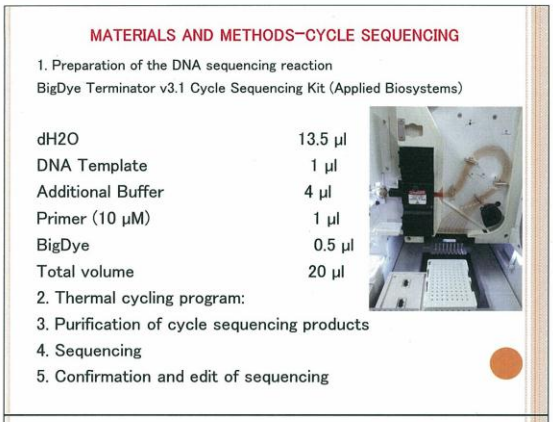
PCR cocktail:

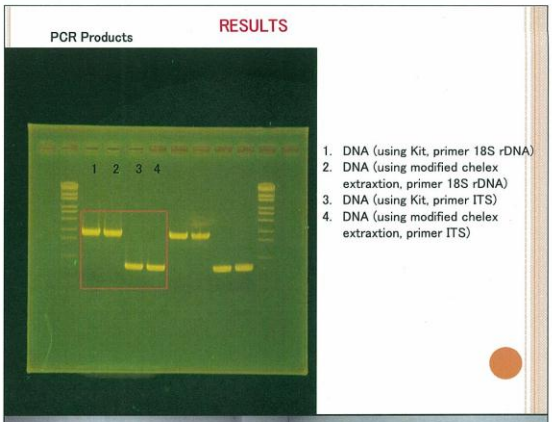
1.	Sterilized distilled water	8.8 µl
2.	2X PCR Buffer	20 µl
3.	dNTPs (2.0 mM each)	8 µl
4.	Forward Primer	0.4 µl
5.	Reverse Primer	0.4 µl
6.	Taq	0.4 µl
7.	DNA Template	2.0 µl
8.	Total volume	40 µl

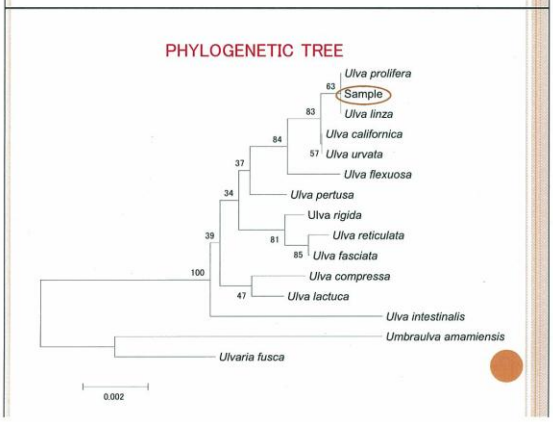

Setting of PCR program: 98ºC: 10s, 60ºC: 30s, 68ºC: 3 mins for 30 cycles

MATERIALS AND METHODS–PURIFICATION OF PCR PRODUCTS

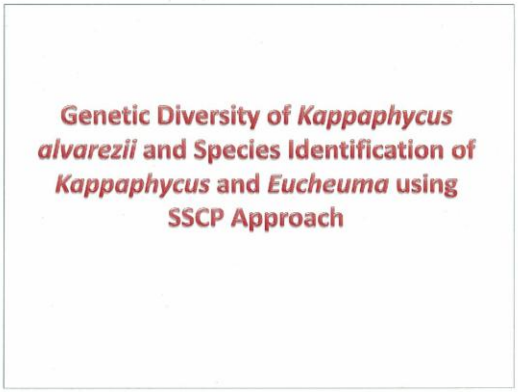
1. PEG solution (PEG 6,000, 20% PEG + 2.5M NaCl) add to the tube of PCR products, vortex, then incubate on ice for 1 hour
2. Centrifuge 14,000 rpm for 15 minutes
3. Remove the PEG supernatant slowly from the tube
4. Wash pellet with 100% ethanol to remove the last traces of PEG
5. Centrifuge 14,000 rpm for 10 minutes then remove ethanol
6. Repeat steps 4 & 5 one more times
7. Dry the pellet for 10 minutes
8. Re-suspend DNA in 50 µl of sterile water

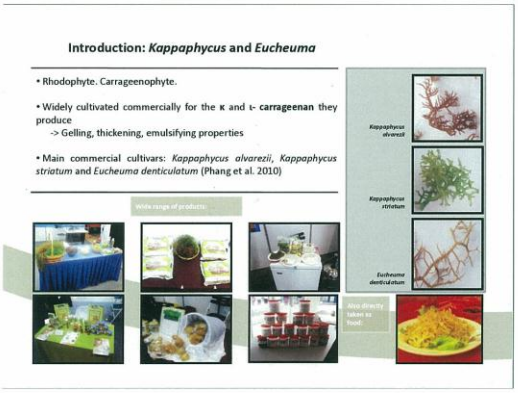



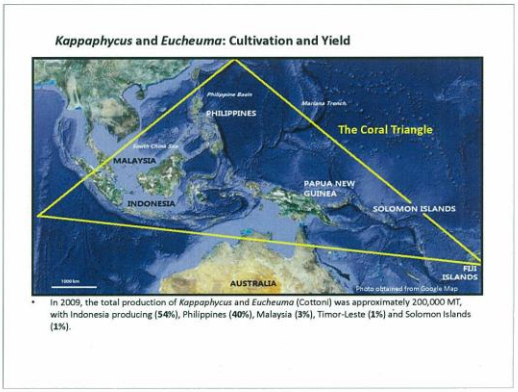




- Phaik Eem LIM (MALAYSIA)







Classification *Kappaphycus* & *Eucheuma*

Phylum	Rhodophyta
Subphylum	Eurhodophytina
Class	Florideophyceae
Subclass	Rhodymeniophycidae
Order	Gigartinales
Family	Solieriaceae

Kappaphycus (currently accepted taxonomically)

Kappaphycus alvarezii (Doty) ex P.C. Silva – type species

Kappaphycus alvarezii var. tambalang (Doty)

Kappaphycus cottonii (Weber-van Bosse) Doty ex P.C. Silva

Kappaphycus inermis (F. Schmitz) Doty ex H.D.Nguyen & Q. N. Huynh

Kappaphycus procrusteanus (Kraft) Doty

Kappaphycus striatus (F.Schmitz) Doty ex P.C.Silva (biosynonym with *Eucheuma striatum* F. Schmitz).

Guiry, M.D. & Guiry, G.M. 2011. *AlgaeBase*

Eucheuma (currently accepted taxonomically)

51 species (and intraspecific) names are found in the database but only 22 have been flagged as currently accepted taxonomically

Type species: *Eucheuma denticulatum* (N.L.Burman) F.S.Collins & Hervey 1917: 106-108

Guiry, M.D. & Guiry, G.M. 2011. *AlgaeBase*

Kappaphycus and *Eucheuma:* Issues and Objectives

Taxonomical confusion

- Numerous varieties of *Kappaphycus* and *Eucheuma*
- Paucity of distinct morphological characters makes identification difficult
- Confusion between local/commercial/scientific names
- *Kappaphycus alvarezii* is the most widely cultivated species in South East Asia due to the high value of **κ -carrageenan**

Objectives:

1. To find out the identities and phylogenetic relationships among *Kappaphycus* and *Eucheuma* varieties – achieved through DNA sequencing (cox 2-3 spacer, *rubisco* spacer)

CAPaBLE

2. The genetic diversity of *Kappaphycus alvarezi i* in South East Asian Countries
3. DNA fingerprint approach (SSCP) in identification of the main cultivated species of *Kappaphycus* and *Eucheuma*

CAPaBLE Training

An Attempt to Search for Suitable Molecular Marker for Genetic Diversity Study

- Primers were designed from *cox*3-*cob* region of mitochondrial genome *Chondrus crispus*

Forward Primers

cox3-R1:5'- TGRGCWTTYTTTCAYAGTAG-3'

cox3-R2:5'- GWTTATCWGATGGKRTTTATGG-3'

Reverse Primers

cob-R1:5'- GCCACCYAATTTATGTGGWATA-3'

cob-R2:5'- CAGTWGCRCCYCATARACTCA-3'

- Only cox3-R2 & cob-R1 primer sets manage to amplify selected *K. alvarezii* samples

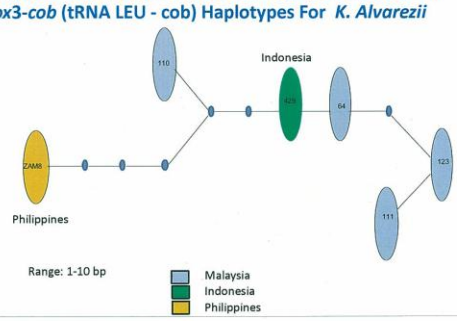
- Based on the partial sequences of *cox*3-*cob* of *K. alvarezii,* new primers set was designed:

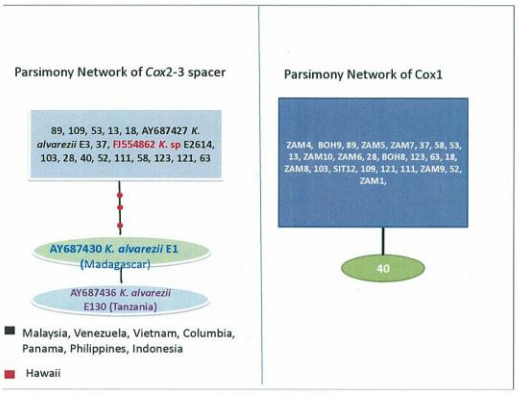
cox3-R3:5'- GCARCATCATTTTGGATTTG-3'

cob-R3:5'- AAACATDGAWGCTCCRTTTG-3'

* Managed to amplify on all the examined *K. alvarezii*

Statistical Parsimony Networks of Partial Mitochondrial *Cox3-cob* (tRNA LEU - cob) Haplotypes For *K. Alvarezii*





Conclusion 1

Partial sequences of tRNA LEU-cob region can potentially be a suitable marker for genetic diversity study on *K. alvarezii*

Single Strand Conformation Polymorphism (SSCP)

- One of the methods used for detection of single point of mutation (Orita *et al.,* 1989).
- Simple and fast
- Working principle: the single stranded DNA frgaments will migrate in a non-denaturing polyacrylamide gel according to their primary nucleotide sequence and not their length.
- SSCP analysis on the *rubisco* spacer was used to detect the variation on red seaweeds such as *Bostrychia* and *Caloglossa* (Zuccarello *et al,* 1999; 2000a; 2000b).

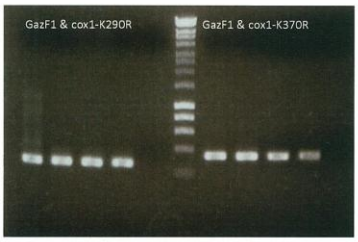
Methodologies

- Two primers were designed based on the *cox*1 sequences of *Kappaphycus* and *Eucheuma*:
  Cox1-K290R: GGAAAAGCCATATCGGGACT
  Cox1-K370R ACACCTACTTCAACCAAAGC
- PCR amplifications:

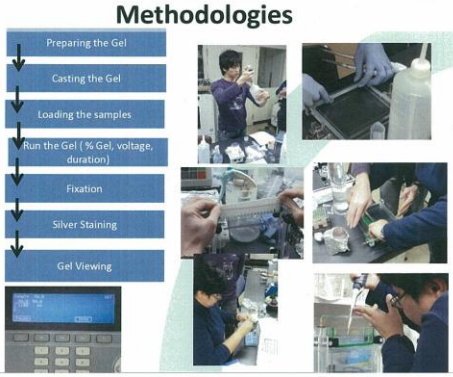
(i) GazF1 & Cox1-K290R

(ii) GazF1 & Cox1-K370R

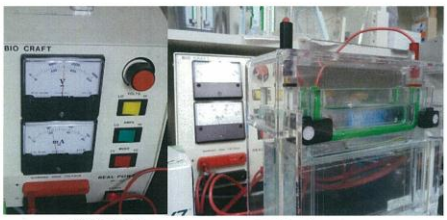
PCR Products



Methodologies

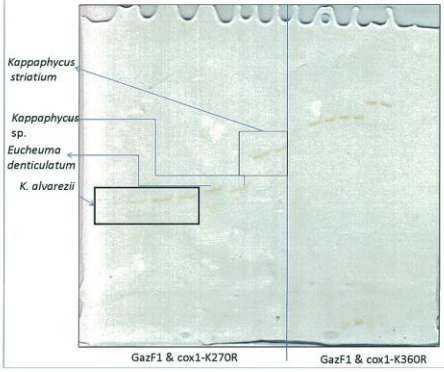


Parameters for SSCP

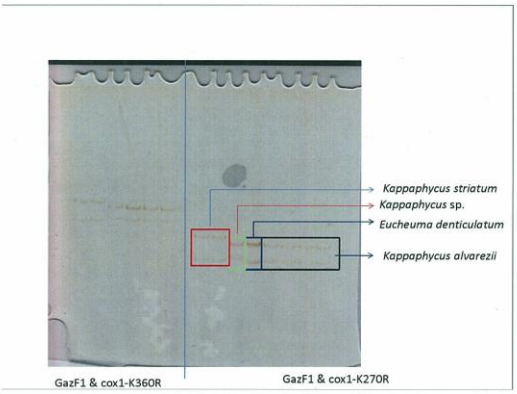


1st Experiment
5% polyacrylamide gel
300V
7 hours

2nd Experiment
2% polyacrylamide gel
200V
7 hours



CBA2011-01CMY-Kawai-FINAL REPORT



Conclusion 2

SSCP of partial *cox*1 sequences can be us as DNA fingerprint method to differentiate different species of *Kappaphycus* and *Eucheuma*

Future Plans

- Will use the tRNALEU – cob region for genetic diversity of *K. alvarezii* from South East Asian countries
- Increased the number of representative species of *Kappaphycus* and *Eucheuma* for the analysis using SSCP of partial *cox*1 sequences.
- SSCP methods can be applied to other Marine invertebrates

Acknowledgement

- APN (Asia Pacific Network)
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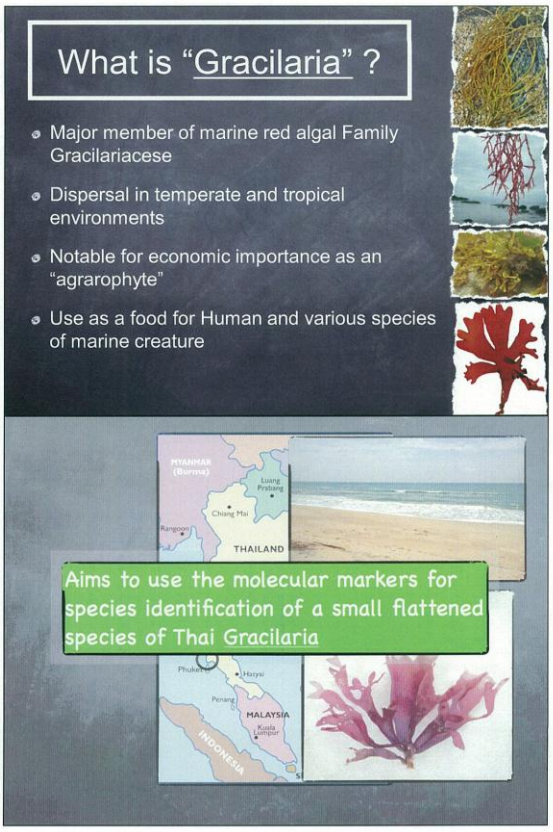
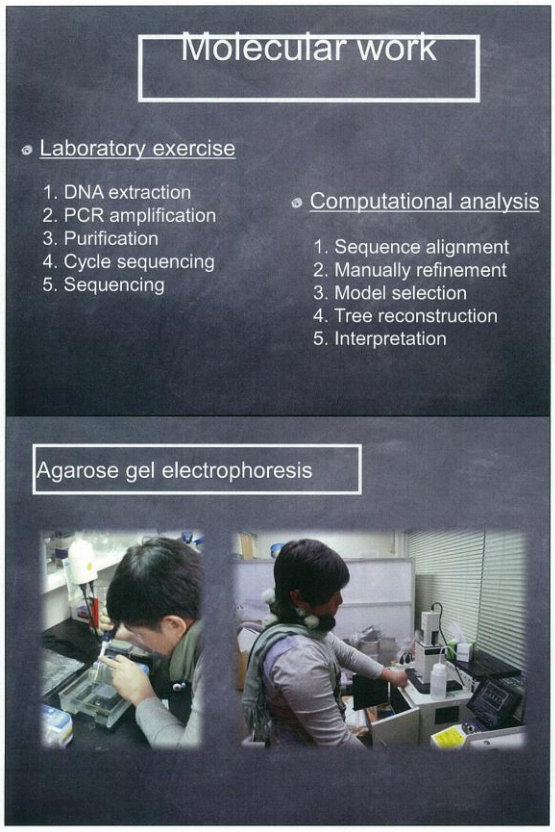
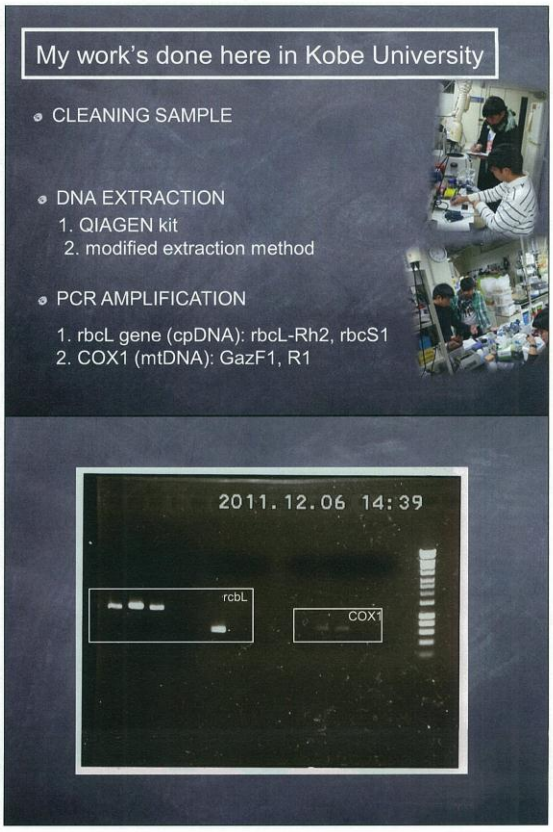
  - Dr. Akira Kurihara
  - Mr. Nazawa
- Consortium of SEASTax: Prof. Phang Siew Moi, Dr. Anicia Hurtado, Prof. Sunarpi, Prof. Dang Diem Hong

- Narongrit MUANGMAI (THAILAND)







- PEG PURIFICATION
- CYCLE SEQUENCING
  rbcL gene: rbcL-Rh2, rbcL-Rh3, rbcL-Rh4, rbcS1
- SEQUENCING

Trimming our DNA data

- BLAST: find region of similarity
- eBioX: multiple alignment
- MacClade: edit and alignment
- Kakusan4: model selection

Build phylogenetic tree!

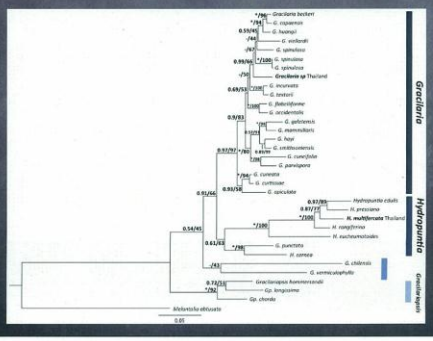
Maximum likelihood tree

- Phylogears
- RAxML

Bayesian tree

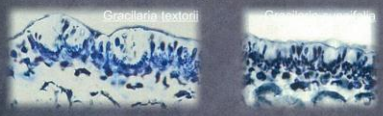
- MrBayes

ML & BI Tree

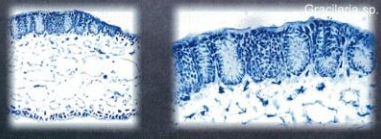


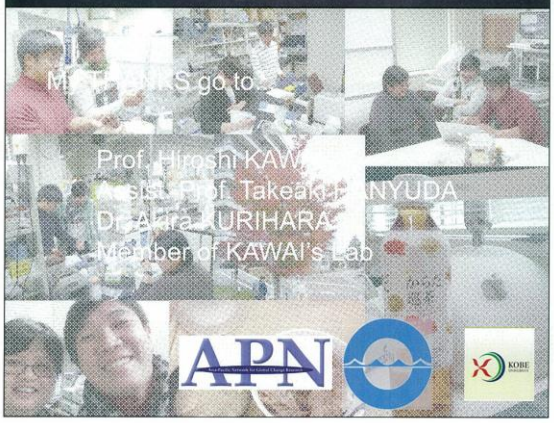
Detailed morphology

- Textorii-type spermatangial conceptacle



- Verrucosa-type spermatangial conceptacle





SUMMARY

- A small flat species of Thai Gracilaria has a close relationship to Gracilaria spinulosa from Taiwan, BUT their male conceptacle configuration are entirely different.
- Base on molecular data and morphology, Thai Gracilaria specimens is POSSIBLE to be distinguished as separate species.
- Molecular techniques are a functional tool for species identification and taxonomic problem resolution.

- Sze-wan POONG (MALAYSIA)

Summary of CAPaBLE Training Programme
3-13 Dec 2011

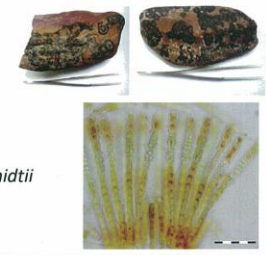
By: Poong Sze Wan
(University of Malaya, Malaysia)

APN KOBE UNIVERSITY HOKKAIDO UNIVERSITY 福井県立大学

BRIEF INTRODUCTION

- Molecular phylogeny of encrusting brown algae (*rbc*L and *cox*1)
- Classification:

Class: Phaeophyceae
Order :Ralfsiales
Family: Mesosporaceae
Genus: *Mesospora*
Species: *Mesospora schmidtii*

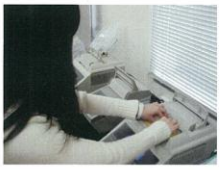


METHODOLOGY

1.DNA EXTRACTION & PURIFICATION WITH STANDARD SUPER-CEL SUSPENSION

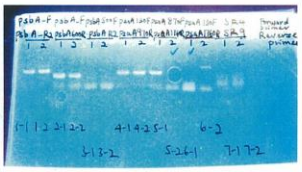
- Extracted DNA from two silica gel-dried samples (*Mesospora schmidtii* 1 and *Mesospora schmidtii* 2)
- Using a low-cost modified method


2. PCR & AGAROSE GEL ELECTROPHORESIS

Two protein-encoding plastid genes
*a) psa*A gene which encodes the PS I P700 chl. A apoprotein A1.
*b) psb*A gene which encodes the PS II thylakoid protein D1.

Primers tested (Yoon et al., 2002):
psbA_F, psbA_R2, psbA600R, psbA _500F;
psaA_130F, psaA_970R, psaA_870F, psaA1760R

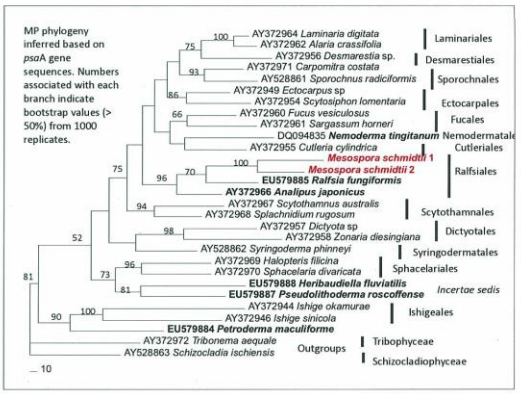
2. PEG PRECIPITATION ,CYCLE SEQUENCING, PURIFICATION OF CYCLE SEQUENCING PRODUCTS

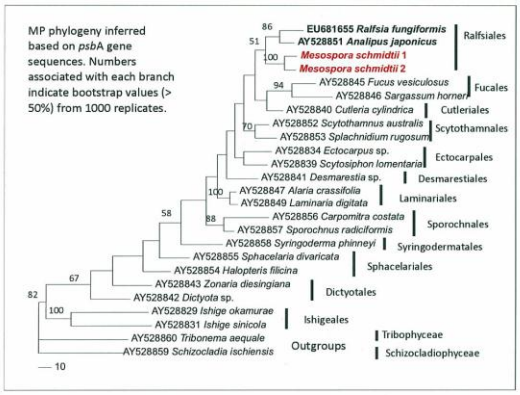
3. ELECTROPHORESIS ON ABI 3730 GENETIC ANALYZER


4. SEQUENCE ALIGNMENT AND PHYLOGENETIC ANALYSES USING PAUP

5. CULTURE OF MICROALGAE: ISOLATION, MEDIUM PREPARATION & SINGLE CELL PCR TECHNIQUE USING FFPE KIT

RESULTS & DISCUSSION





ACKNOWLEGDEMENT

Practical training instructors:

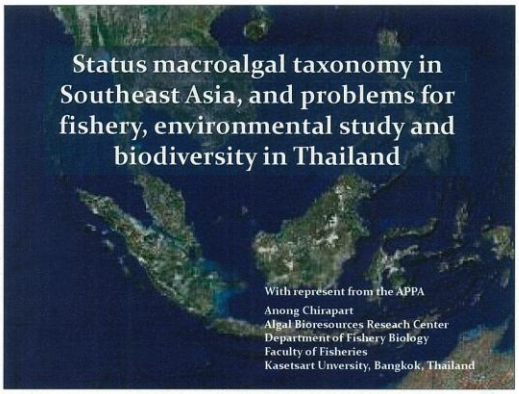
Prof. Takeo Horiguchi

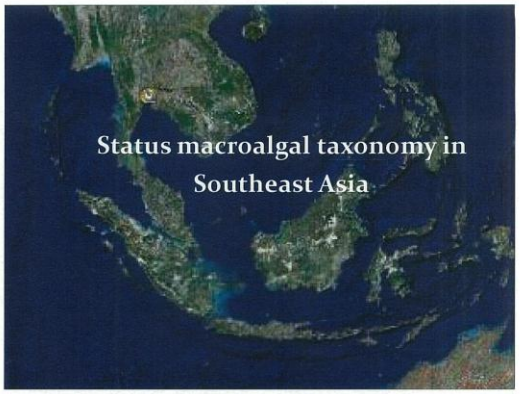
Prof. Kazuhiro Kogame

The Phycological Laboratory, Faculty of Science, Hokkaido University

Appendix 8 Power Point Slides of Invited Commentator and Project Leader at Summary Workshop

2010 Anong CHIRAPART (KASETSART UNIVERSITY)





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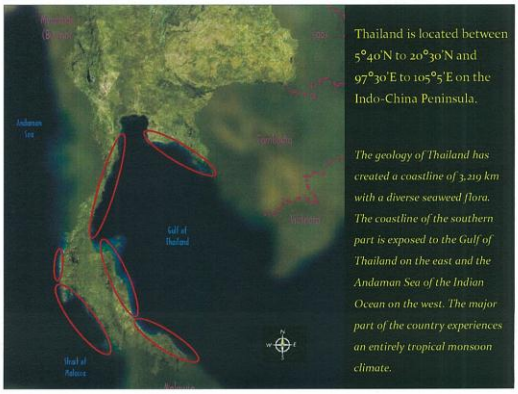
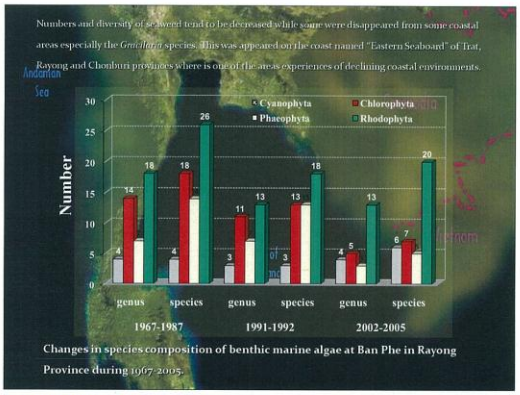
Algal taxonomy: Problems for biodiversity, environmental study and fishery in Thailand

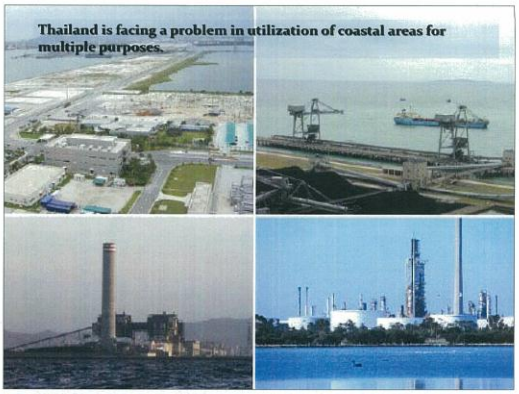


Thailand is located between 5°40'N to 20°30'N and 97°30'E to 105°5'E on the Indo-China Peninsula.

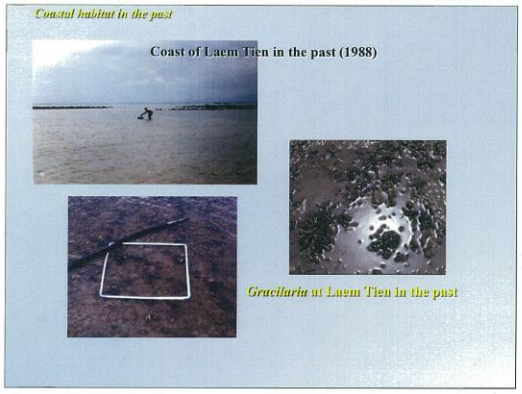
The geology of Thailand has created a coastline of 3,219 km with a diverse seaweed flora. The coastline of the southern part is exposed to the Gulf of Thailand on the east and the Andaman Sea of the Indian Ocean on the west. The major part of the country experiences an entirely tropical monsoon climate.

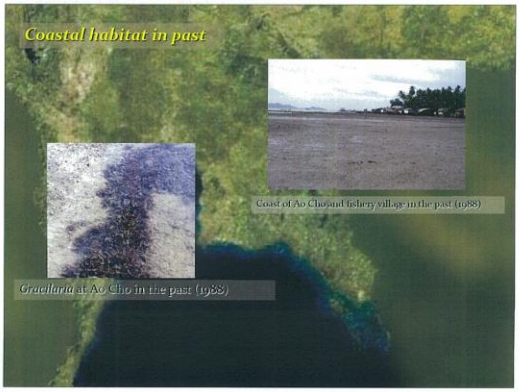


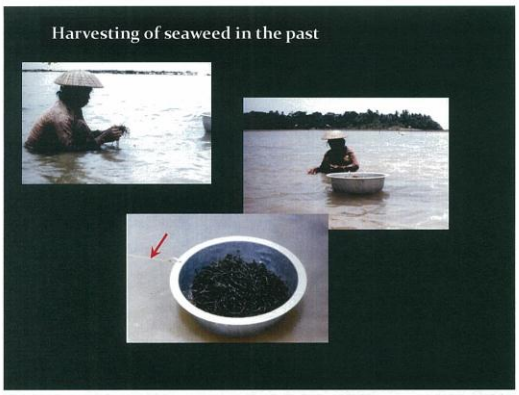
Changes in species composition of benthic marine algae at Ban Phe in Rayong Province during 1967-2005.

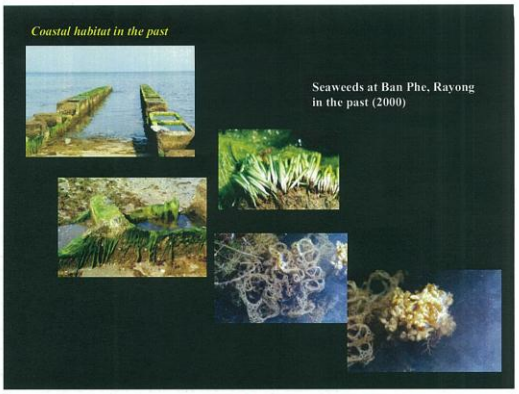


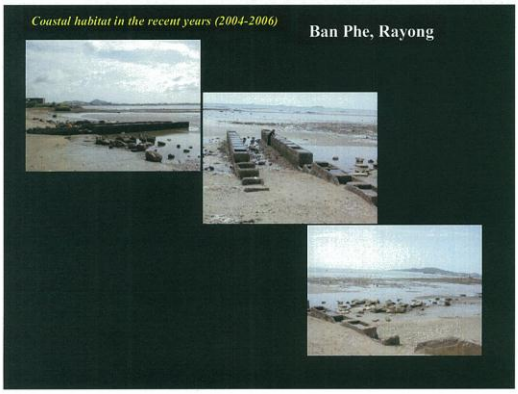
Thailand is facing a problem in utilization of coastal areas for multiple purposes.

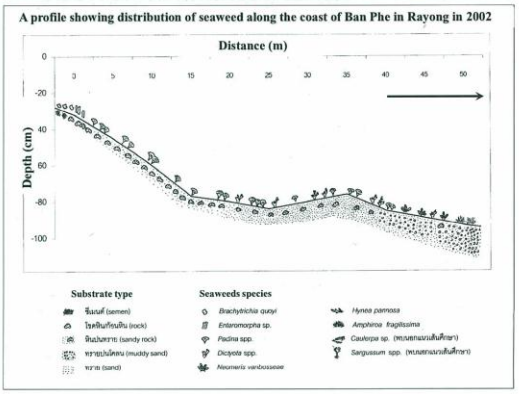


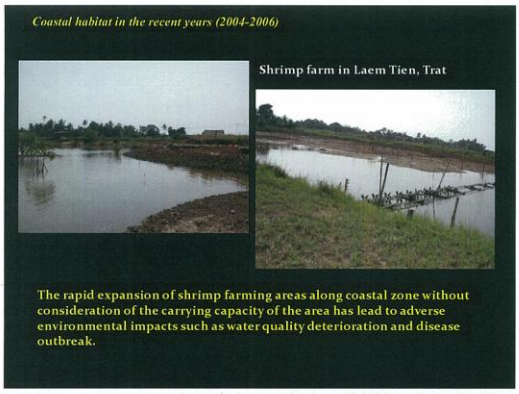


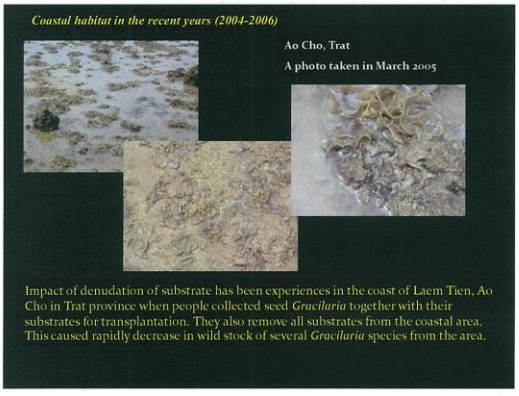


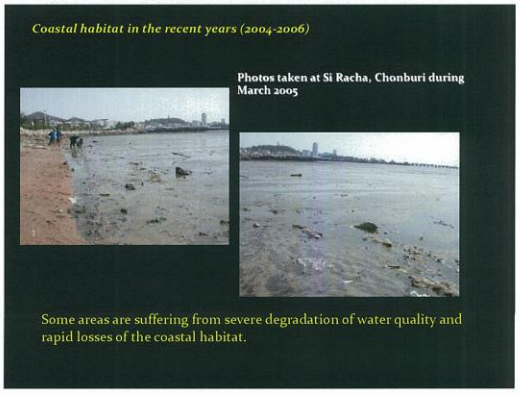


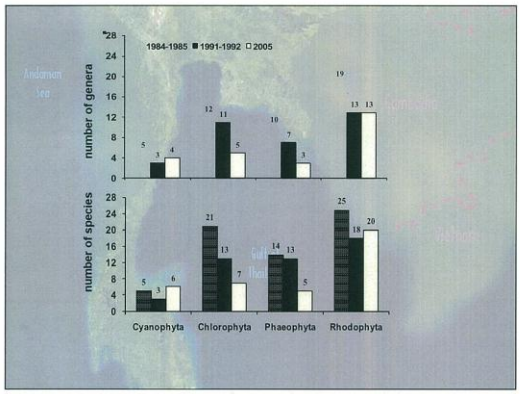


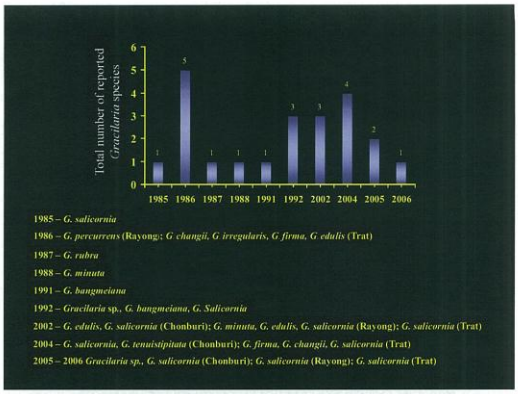


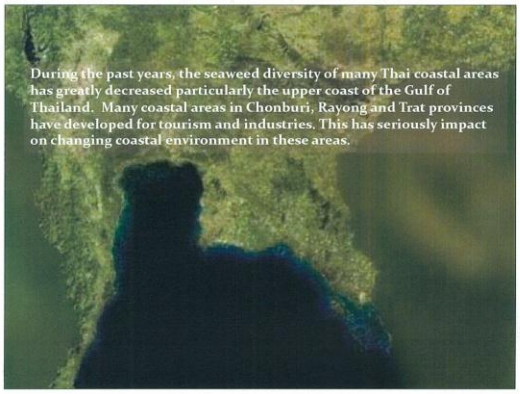


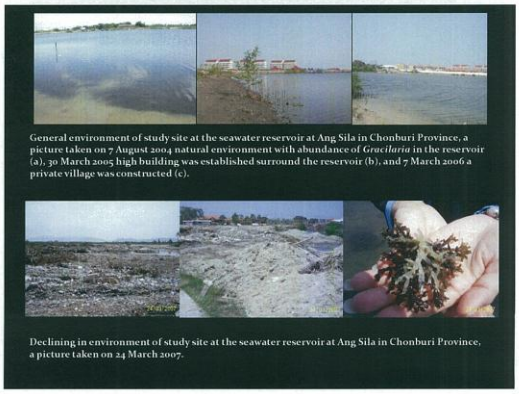


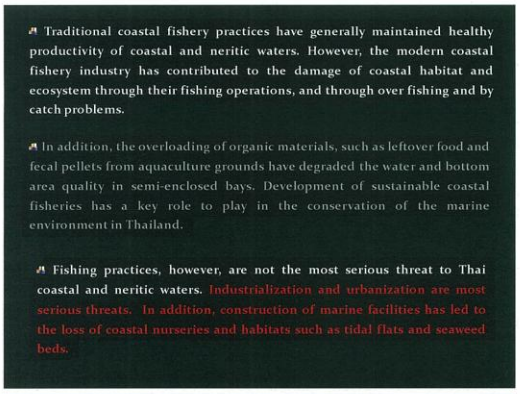



1984-1985 1991-1992 2005
number of genera
number of species
Cyanophyta
Chlorophyta
Phaeophyta
Rhodophyta


Total number of reported Gracilaria species
1985 1986 1987 1988 1991 1992 2002 2004 2005 2006
1985 – G. salicornia
1986 – G. percurrens (Rayong); G. changii, G. irregularis, G. firma, G. edulis (Trat)
1987 – G. rubra
1988 – G. minuta
1991 – G. bangmeiana
1992 – Gracilaria sp., G. bangmeiana, G. Salicornia
2002 – G. edulis, G. salicornia (Chonburi); G. minuta, G. edulis, G. salicornia (Rayong); G. salicornia (Trat)
2004 – G. salicornia, G. tenuistipitata (Chonburi); G. firma, G. changii, G. salicornia (Trat)
2005 – 2006 Gracilaria sp., G. salicornia (Chonburi); G. salicornia (Rayong); G. salicornia (Trat)


During the past years, the seaweed diversity of many Thai coastal areas has greatly decreased particularly the upper coast of the Gulf of Thailand. Many coastal areas in Chonburi, Rayong and Trat provinces have developed for tourism and industries. This has seriously impact on changing coastal environment in these areas.


General environment of study site at the seawater reservoir at Ang Sila in Chonburi Province, a picture taken on 7 August 2004 natural environment with abundance of Gracilaria in the reservoir (a), 30 March 2005 high building was established surround the reservoir (b), and 7 March 2006 a private village was constructed (c).
Declining in environment of study site at the seawater reservoir at Ang Sila in Chonburi Province, a picture taken on 24 March 2007.


Traditional coastal fishery practices have generally maintained healthy productivity of coastal and neritic waters. However, the modern coastal fishery industry has contributed to the damage of coastal habitat and ecosystem through their fishing operations, and through over fishing and by catch problems.
In addition, the overloading of organic materials, such as leftover food and fecal pellets from aquaculture grounds have degraded the water and bottom area quality in semi-enclosed bays. Development of sustainable coastal fisheries has a key role to play in the conservation of the marine environment in Thailand.
Fishing practices, however, are not the most serious threat to Thai coastal and neritic waters. Industrialization and urbanization are most serious threats. In addition, construction of marine facilities has led to the loss of coastal nurseries and habitats such as tidal flats and seaweed beds.


Research on algal taxonomy in combination with other research
gather environmental data for ecological and distributional studies
Morphological investigation of seaweed: morphometric variation
Molecular biology techniques: to reinforce the shortage of traditional morphological approach
cell biology techniques: to reinforce the shortage of traditional morphological approach

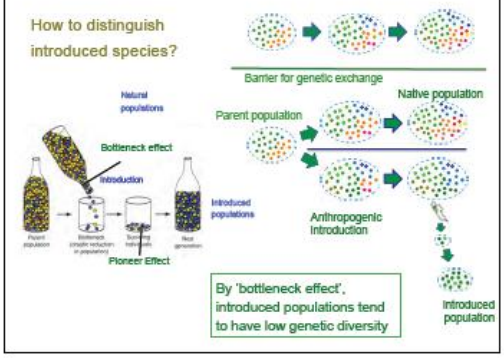

Thank you for yours attention

2011 Hiroshi KAWAI (KOBE UNIVERSITY)

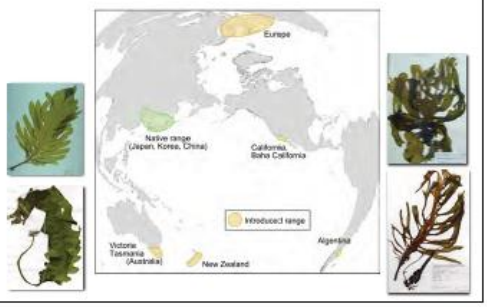
Introductions (?) of marine organisms

1. True expansion of distributions
   A) Associated with natural phenomena (e.g. current)
   B) Artificial introduction
      - Intentional introduction and non-intentional contamination associated with the introduction
      - Introduction associated with ship transfer
      - Contamination associated with sea sand transport
2. Increase and emergence of native species by environmental changes (e.g. eutrophication, pollution, climate change, etc.)
3. Increase of knowledge associated with increasing interests and researches

How to distinguish introduced species?

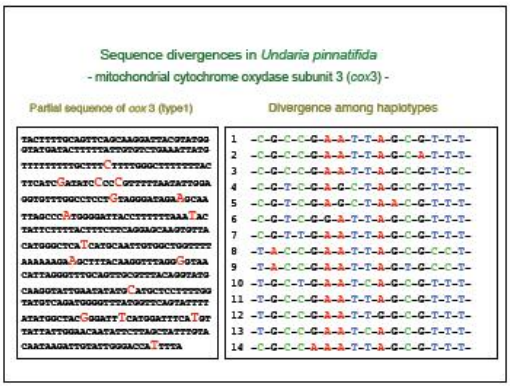


Worldwide distribution of *Undaria pinnatifida*

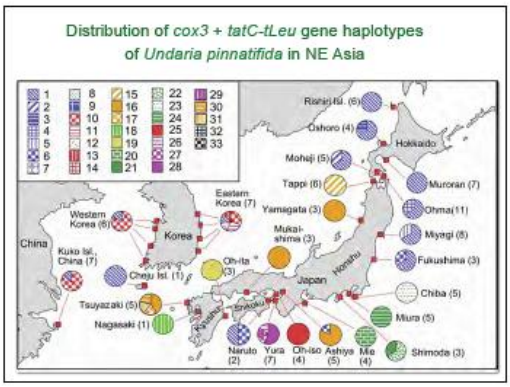


First records of *U. pinnatifida* populations in New Zealand

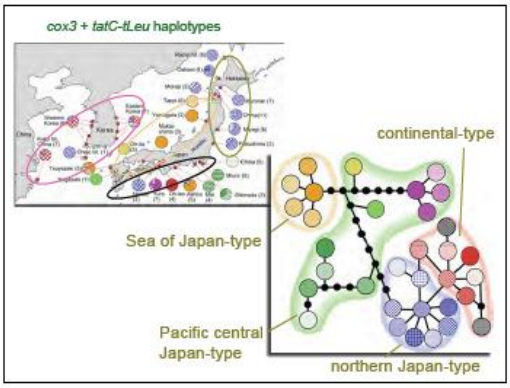
Sequence divergences in *Undaria pinnatifida*
- mitochondrial cytochrome oxydase subunit 3 (*cox3*) -



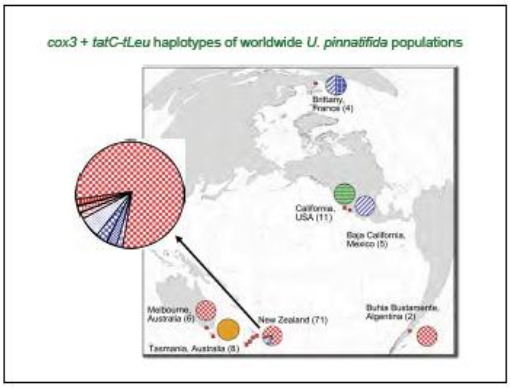
Distribution of *cox3* + *tatC-tLeu* gene haplotypes of *Undaria pinnatifida* in NE Asia

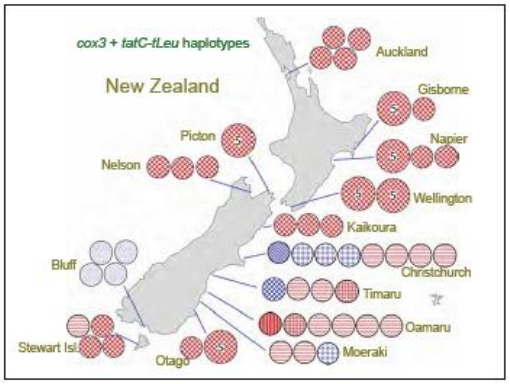


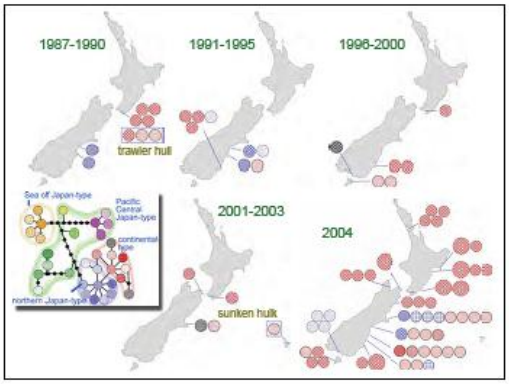
cox3 + *tatC-tLeu* haplotypes

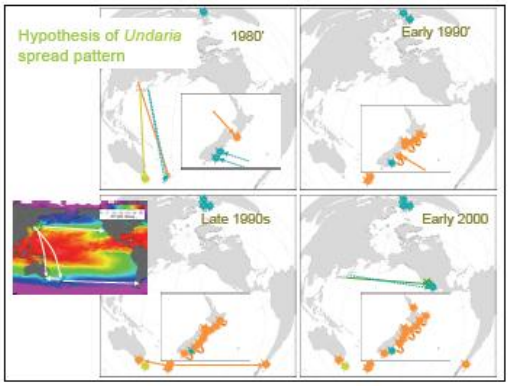


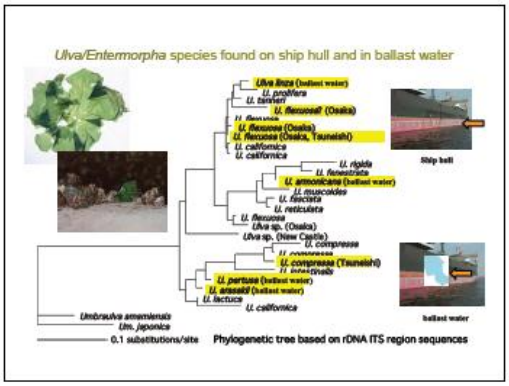
cox3 + *tatC-tLeu* haplotypes of worldwide *U. pinnatifida* populations

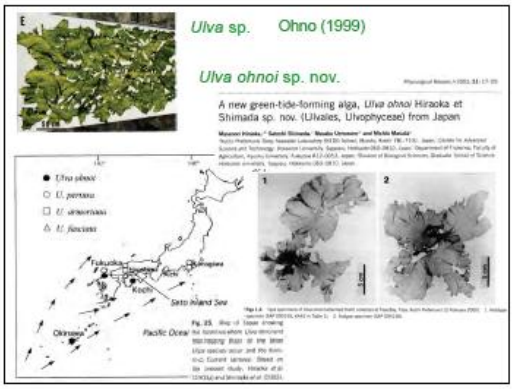


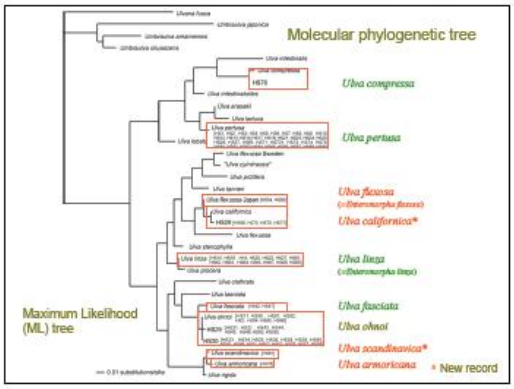


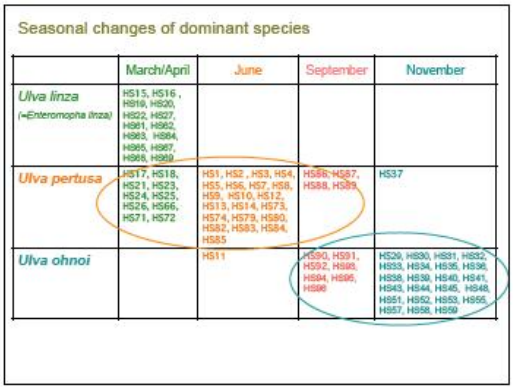




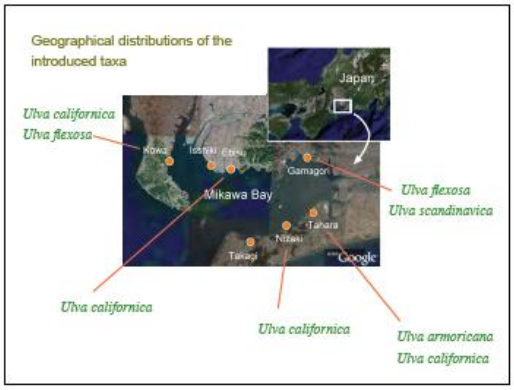


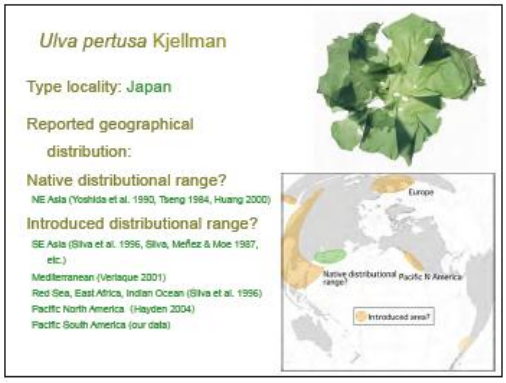


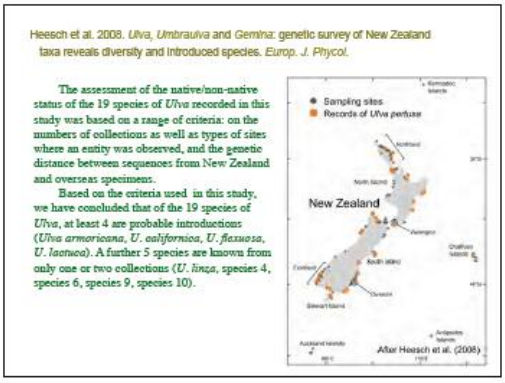


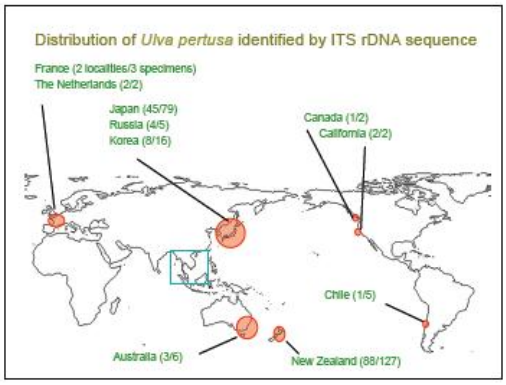


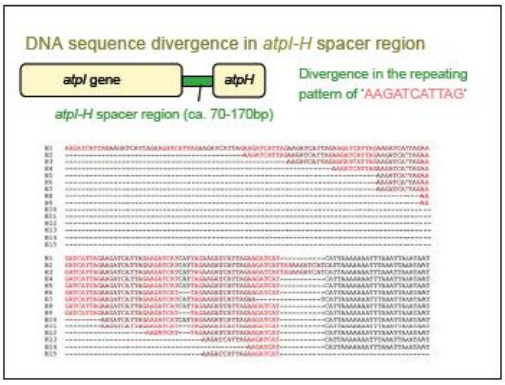
	March/April	June	September	November
Ulva linza (=*Enteromopha linza*)	HS15, HS16, HS19, HS20, HS22, HS27, HS81, HS82, HS83, HS84, HS85, HS87, HS88, HS89			
Ulva pertusa	HS17, HS18, HS21, HS23, HS24, HS25, HS26, HS66, HS71, HS72	HS1, HS2, HS3, HS4, HS5, HS6, HS7, HS8, HS9, HS10, HS12, HS13, HS14, HS73, HS74, HS79, HS80, HS82, HS83, HS84, HS85	HS86, HS87, HS88, HS89	HS37
Ulva ohnoi		HS11	HS90, HS91, HS92, HS95, HS94, HS95, HS96	HS26, HS30, HS31, HS32, HS33, HS34, HS35, HS36, HS38, HS39, HS40, HS41, HS43, HS44, HS45, HS48, HS51, HS52, HS53, HS55, HS57, HS58, HS59

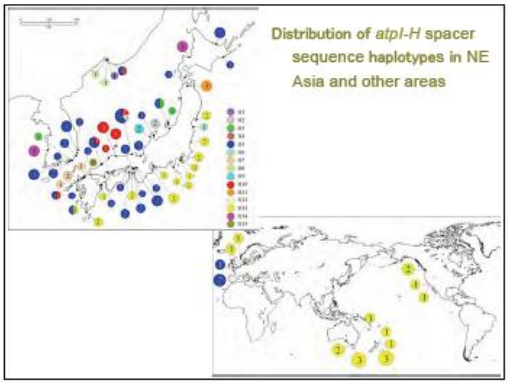


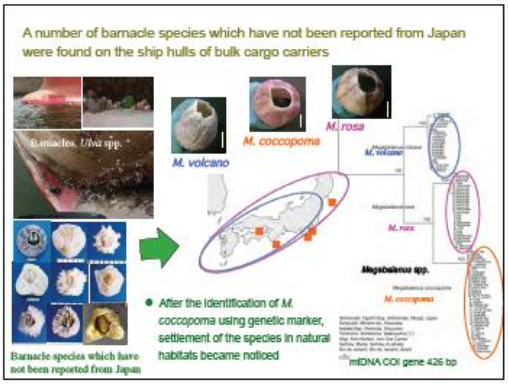


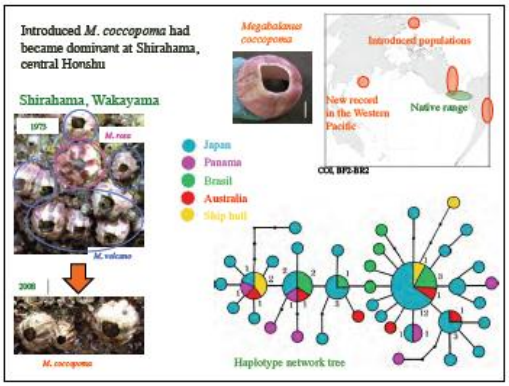


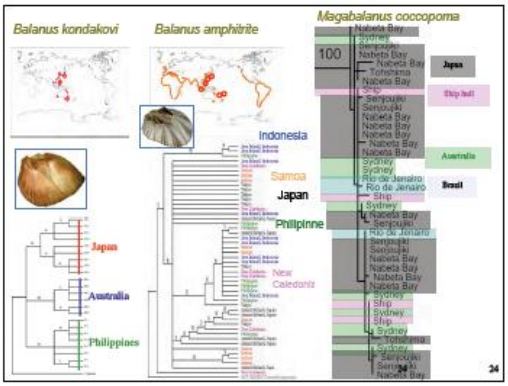












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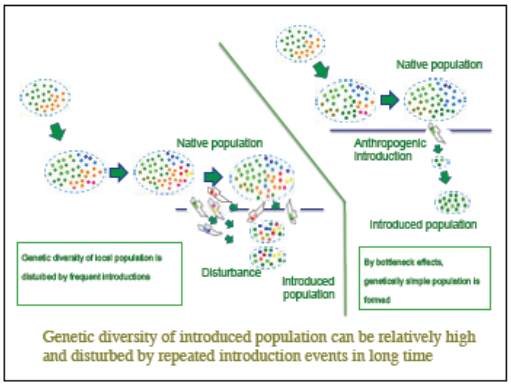


Genetic diversity of introduced population can be relatively high and disturbed by repeated introduction events in long time

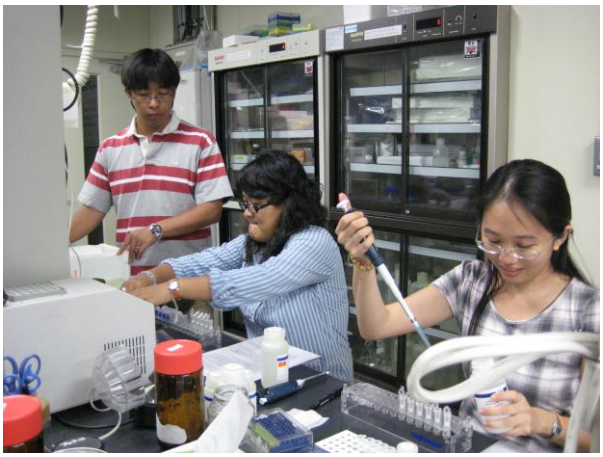
Appendix 9 Pictures during Training

2010

Introductory Meeting (Jul. 4th, 2010)

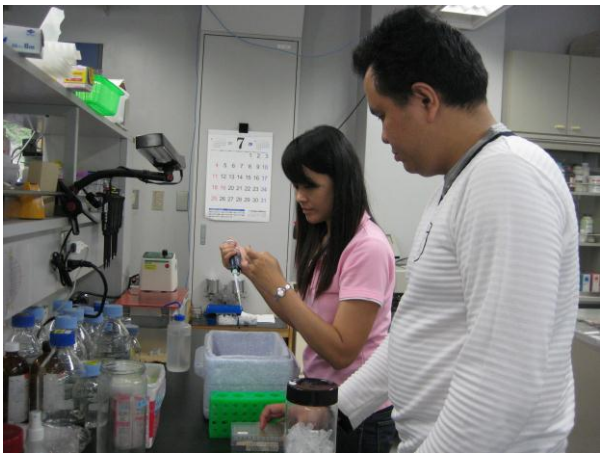
Introductory Meeting (Jul. 4th, 2010)

Group photo at Introductory Meeting



Practical Training at Kobe University

Practical Training at Hokkaido University



Practical Training at Fukui Pref. University

Interview with reporter for local newspaper
(Fukui Newspaper)

Summary Meeting (Jul. 12th, 2010)

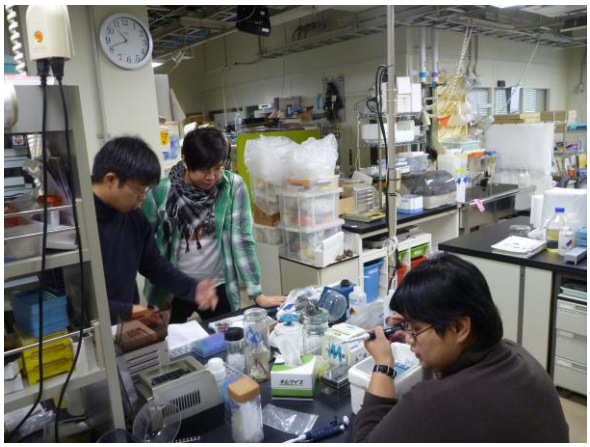
Summary Meeting; Comments from Guest

Group photo at Summary Meeting

2011

Introduction Meeting (Dec. 4th, 2011)

Group Photo at Introduction Meeting



Practical Training at Kobe University

Practical Training at Hokkaido University

Practical Training at Fukui Prefectural University

Summary Meeting (Dec. 11th, 2011)

Group Photo at Summary Meeting

Reception (Dec. 11th, 2011)

Instructors

Project Leader; Prof. Kawai

Collaborator; Prof. Horiguchi

Collaborator; Prof. Kamiya

Collaborator; Dr. Kogame

Instructor; Dr. Hanyuda

Instructor; Dr. Kurihara

Invited Commentator

Invited Commentator; Prof. Chirapart

Host Laboratories

Kobe University

Hokkaido University

Fukui Prefectural University

Organizer

Closing Remark from APN
Director; Mr. Fujitsuka

Closing Remark from EMECS
Executive Director; Mr. Arashi

The report about the CAPaBLE Training on the local newspaper, “Fukui Newspaper”
(Jul. 10, 2010)

福井新聞
(27) 地域 2010年(平成22年)7月10日(土曜日)

嶺南

ニュースなど ご連絡は
敦賀支社 ☎0770(22)3939 FAX(22)4529
小浜支社 ☎0770(52)3311 FAX(52)3312

夢風船

★パティシエになりたい★
お菓子を作るのが好きなので、パティシエになりたいです。わたしが作ったケーキやクッキーを食べてもらうと、みんな「おいしい」と言ってくれます。もっとおいしいお菓子を作りたいです。美浜町美浜北小6年、片山日奈さん

日本の設備で学んで
アジアの研究者受け入れ
県立大

県立大は5日から同大、究者に日本の設備を使っム（CAPaBLE）の小浜キャンパスでアジアて研究手法を学んでもら一環として本年度から始太平洋地域の若手研究者おうと、財団法人国際エめた。
2人を受け入れた。研究メックスセンター（神戸能力向上に向けた研修を市）が企画。アジア太平9日まで実施した。洋地球変動研究ネットワ
受け入れは、設備不足ーク（APN）の助成を2人ずつ受け入れた。県などで十分な研究がで立大にはインドネシアのきないアジアの若手研能力開発・向上プログラ ムラトランギ大講師ロ

藻類の分類研究に励むフィムラさん（左）、モントラルさん（中央）と神谷准教授＝県立大小浜キャンパス

イケ・イワン・モントラルさん（37）とタイのカセサート大研究員のソラタコーン・フィムラさん（29）が訪れ、海洋生物資源学部の神谷充伸准教授の指導を受けた。

2人が取り組んだのは藻類の分類研究。2人は亜熱帯の海域にみられる海藻を持参し、これら種のものかなどを調べた。2人は「研究設備が整っている」、研究手法も学べて勉強になる。今回学んだことを今後の研究で生かしたい」と話していた。