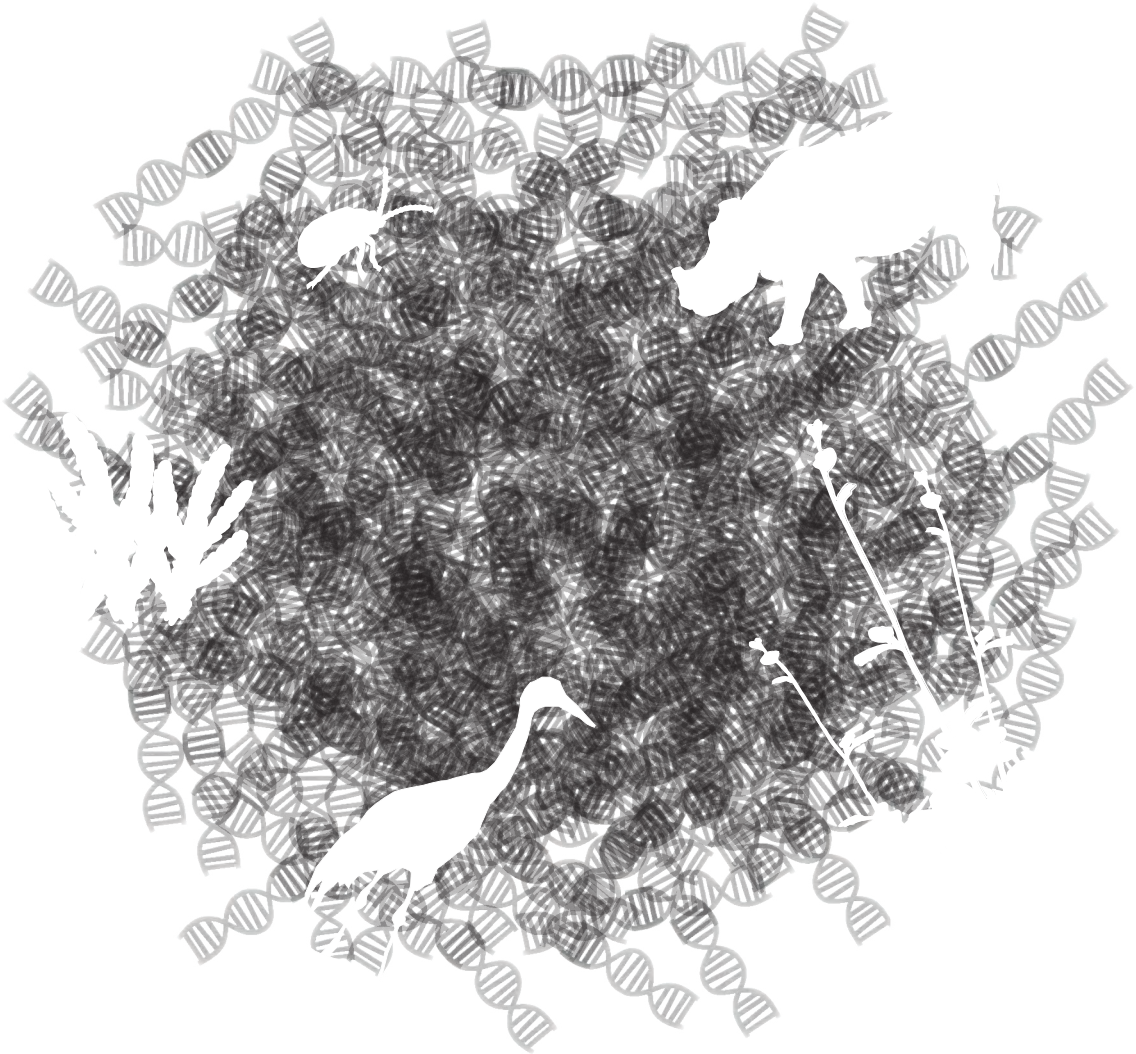
Center for Environmental Biology and Ecosystem Studies, National Institute for Environmental Studies

Symposium

Unraveling Biodiversity from DNA

- From the Management of Databases to the Use of Next Generation Sequencers -



Sept. 19th, 2014

Tsukuba, Japan

Cover design: Kiyono Katsumata; Center for Environmental Biology and Ecosystem Studies, National Institute for Environmental Studies (NIES)

Welcome Message

On behalf of the Organizing Committee, I am pleased to invite you to the international symposium, entitled ‘Unraveling biodiversity from DNA – from the management of databases to the use of next generation sequencers’- at the National Institute for Environmental Studies (NIES), Tsukuba, Japan.

In recent years we have been able to acquire massive quantities of nucleotide sequences with relative ease. These nucleotide sequences are useful indicators to identify living organisms and detect their genetic changes and gene expression level, etc, and biological studies using the nucleotide information are proceeding apace. These new methods can be expected to facilitate innovations in our current researches on biodiversity conservation, preservation of biological resources and long-term environmental monitoring. I believe that this symposium will provide a good opportunity to learn more about recent advances in the knowledge of wildlife conservation, DNA barcoding, and biodiversity studies using next generation sequencing, and will open a new frontier in biodiversity conservation research with the current biological DNA studies.

For this symposium, we have invited four distinguished researchers from abroad and seven active Japanese researchers some of whom are affiliated with the Center for Environmental Biology and Ecosystem Studies at NIES. In addition, 23 poster presentations have been prepared. Active discussions from the floor are welcomed, such that we might better understand these pioneering studies. I hope you enjoy the symposium.

Noriko Takamura

Director, Center for Environmental Biology and Ecosystem Studies,

National Institute for Environmental Studies

Program 19 September, 2014

Opening remarks

9:10–9:20 Akimasa Sumi (President, NIES, Japan)

Session I. Wildlife

Chairperson: Dr. Manabu Onuma

9:20–10:00 Oliver A. Ryder (San Diego Zoo Institute for Conservation Research, USA)
"Genetic Rescue of small populations: an emerging role for genetic resource collections"

10:00–10:40 Rob Ogden (Royal Zoological Society of Scotland, Edinburgh, UK)
"The role of genomics in wildlife conservation: real solutions or academic exercise?"

10:40–10:50 Break

10:50–11:20 Miho Murayama (Kyoto University; NIES, Japan)
"Application of DNA/cell database for wildlife conservation"

11:20–11:50 Nobuyoshi Nakajima, Manabu Onuma (NIES, Japan), Daiji Endo (Rakuno Gakuen University, Japan)
"Establishment of genome databases for the protection of endangered species"

11:50–13:50 Lunch and Poster

Session II. DNA barcoding and Databases

Chairperson: Dr. Masanobu Kawachi

13:50–14:20 Manabu Onuma (NIES, Japan)
"Establishing a genetic resource bank for Asian endangered species and its application for conservation"

14:20–15:00 Torbjørn Ekrem (NTNU University Museum, Norway)
"NorBOL - the Norwegian Barcode of Life Initiative"

15:00–15:30 Natsuko I. Kondo, Ryuhei Ueno, Kenzi Takamura (NIES, Japan)
"DNA barcoding of Japanese chironomids"

15:30–16:00 Keiko Kishimoto-Yamada, Motomi Ito (University of Tokyo, Japan)
"DNA barcoding plant-insect interactions and activities of Japanese Barcode of Life Initiative (JBOLI)"

16:00–16:10 Break

Session III. Biodiversity and Next Generation Sequencing

Chairperson: Dr. Yayoi Takeuchi

16:10–16:50 Todd C. LaJeunesse (Penn State University, USA)
"The expanding role of next generation sequencing in the research of coral-dinoflagellate symbioses"

16:50–17:20 Shosei Kubota (Nihon University, Japan)
"Dynamics of local adaptation from standing genetic variation in a wild *Arabidopsis* species"

17:20–17:50 Atsushi J. Nagano (Kyoto University, Japan)
"Deciphering and prediction of transcriptome dynamics in a real world"

Closing remarks

17:50–18:00 Noriko Takamura (Director of Center for Environmental Biology and Ecosystem Studies, NIES, Japan)

Reception Party

18:30–20:30 NIES Canteen

Session I: Wild Life
9:20–10:00

Genetic Rescue of small populations: an emerging role for genetic resource collections

Oliver A. Ryder

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Rapidly decreasing costs for DNA sequencing and advances in genome assembly and analysis are producing an unprecedented expansion of biological information that will re-shape biological disciplines and impact all aspects of biological inquiry. Whole genome sequence information is becoming available for endangered species and their close relatives, offering new opportunities for evaluating genetic diversity and identifying the genetic basis for adaptations. Revealing the evolutionary history of species, recognizing gene flow and hybridization, detection of population bottlenecks and expansions, and identification of loci under selection, affecting disease susceptibility and life history variables have become the subjects of studies that offer the promise of informed interventions to rescue species from extinction (1). Combined with advanced genetic technologies and the judicious banking of samples of cells and gametes, restoration of lost genetic diversity to improve population sustainability can contribute to biodiversity conservation efforts. The San Diego Zoo's Frozen Zoo® is a repository of viable frozen somatic cells, gametes, and tissues that contributes to genome sequencing efforts, notably through the Genome10K project (2), and can serve as a source for restoring genetic variation in critically small populations of vertebrates. Examples of ongoing studies of black-footed ferrets, California condors, great apes, and Mississippi gopher frogs serve as current examples of conservation genomics and genetic rescue projects in our laboratory.

References:

1. Steiner et al. (2013) Annual Review of Animal Biosciences 1, 261–81.
2. Haussler et al. (2009) J. Hered. 100, 659-674.

Session I: Wild Life
10:00–10:40

The role of genomics in wildlife conservation: Real solutions or academic exercise?

Rob Ogden

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The application of molecular genetic data to wildlife conservation, known generally as Conservation Genetics, has been an established discipline for over 15 years. However it can be argued that despite its name, the field has delivered relatively little practical support to conservation programmes and has instead largely become an area of academic study.

The past five years has seen the rapid uptake of genomic era technologies by the conservation genetic community. During the same period we have seemeingly entered a new phase in the battle against the illegal wildlife trade and the threats to biodiversity through human-mediated environmental change.

The increasing availability of whole genome sequences, genome-wide SNP genotyping approaches and user-friendly bioinformatic pipelines is starting to bring the potential of genomics within the reach of conservation biologists. The academic community is promising much, from greater resolution of classical population genetic issues such as geographic structure, gene flow and hybridisation history to a first real understanding of the genes involved in local adaptation and natural selection in non-model wildlife species.

This paper will focus on the applied uses of genomics in wildlife conservation to examine if these technologies are actually starting to provide novel data to conservation practitioners or whether the genomic revolution has really only widened the gap between academia and wildlife management. If so, how can the scientific community adapt its research model to the needs of the conservation community?

Session I: Wild Life
10:50–11:20

Application of DNA/cell database for wildlife conservation

Miho Murayama

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To learn more about animals we have been focusing on molecular-based approaches based on analysis on DNA/cell or hormone. The outcomes of our study will not only benefit captive animals in zoos and aquariums but also provide useful information for conservation of endangered species in the wild. To understand the range of genetic diversity within species requires obtaining and genotyping many samples for each species. We have collected DNA samples from over 24,000 individuals representing 400 species of both mammals and birds. Since some animals were difficult to obtain samples directly, we devised methods for efficient analysis using noninvasive samples such as feces and hair. We store these data in a DNA database (the DNA Zoo), which also includes the geographical information of the collected samples and the characteristics of the individual. We are going to develop the DNA Zoo, so that it is able to link to cell bank. Using the DNA Zoo, we can identify subspecies/populations/kinships/individuals/sex by genotyping polymorphic markers such as microsatellites and mitochondrial DNA. Introduction of the new techniques, such as next-generation sequencing, made marker isolation, food-item survey and meta-genome analysis easier. We also conduct a comparative analysis of molecular basis and observation of individual behavior differences related to stress susceptibility, social relationship and reproduction. For example, we can investigate behavior of an individual through observation, hormone level, genetic background and cellular function. These approaches widen the possibility of molecular ecology of wild animals.

References:

1. Inoue-Murayama (2009) Animal Science Journal 80, 113–120.
2. Inoue-Murayama et al. (eds.) (2012) From Genes to Animal Behavior. Springer, Tokyo.

Session I: Wild Life
11:20–11:50

Genome sequencing of endangered avian species

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Human activities have accelerated extinction of biological species. In the history of life, numerous rises and falls of species have taken place. Species extinction used to take place at an average rate of no more than 10 species per year, often due to environmental changes resulting from inevitable events such as the meteorite impact and the glacial expansion. After the World War II, however, more than 10,000 species have been disappearing every year (1).

Besides protecting endangered species, storage of their genetic resources seems important. Because DNA is vulnerable to environmental radiation and it is impossible to keep DNA intact for a long time without biological replication and repair processes, acquisition of the nucleotide sequences rather than preserving the DNA itself is desirable. Resent advance in sequencing technology significantly reduced the cost for whole-genome sequencing and genomes of increasingly many species (eg. more than 150 vertebrates) are being sequenced. Whole genome data provide us at least 2 benefits for researches on extinct and endangered species;

(A) Morphologically indistinguishable species can be identified. Survey of species distribution based on accurate taxonomic classification is very important for the protection of endangered species.

(B) Physiological and ecological characteristics of extinct and endangered species can be deduced from genomic sequence comparison with other species.

Currently we are analyzing the whole genome structures of three endangered avian species (*Gallirallus okinawae*, *Ciconia boyciana*, *Grus japonensis*) to store their genetic resources, to understand their life cycles, and to improve the tactics for species protection.

Reference:

1. Myers and Kent (2005) The sinking ark: a new look at the problem of disappearing species. University of California Press.

Session II: DNA barcoding and Databases
13:50–14:20

Establishing a genetic resource bank for Asian endangered species and its application for conservation

Manabu Onuma

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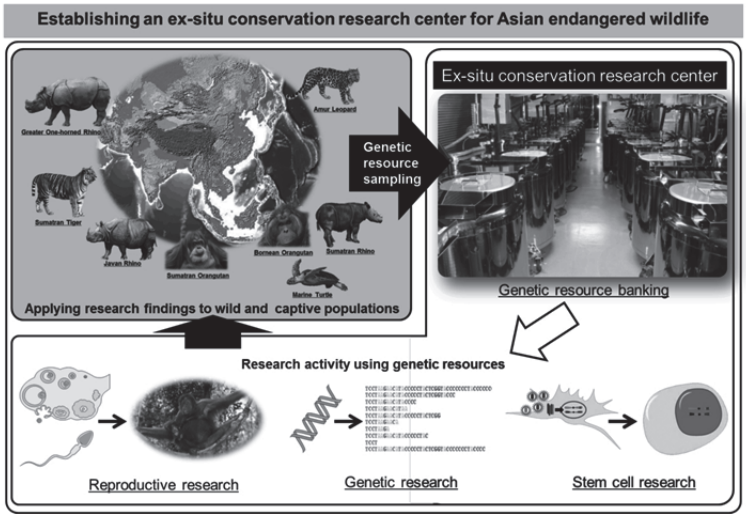
There are two main strategies for wildlife conservation, namely, ex situ and in situ conservation. A genetic resource bank (cryopreserving genetic materials and live cells) is one of the options for ex situ conservation. The famous example of this option is known as the "Frozen Zoo," which have been established in San Diego Zoo. In Japan in 2002, the National Institute for Environmental Studies (NIES) also established a resource bank of this type to start cryopreserving the genetic materials of endangered species in Japan: an intiative referred to as the "Time capsule project."

NIES has also attempted to expand the genetic resouce banking project to other countries in the Asian region. The new project, "Establishing an ex-situ conservation research center for Asian endangered wildlife," which started in 2011, aims to establish a genetic resource bank network for endangered species in the Asian region and to promote effective utilization of genetic resources for conservation purposes. Five related authorities in Nepal, Indonesia, Malaysia, Singapore, and Russia will join the network. There are currently five priority wildlife categories, namely, Asian rhinoceros, orangutans, tigers, leopards, and marine turtles.



Session II: DNA barcoding and Databases
14:20–15:00

NorBOL - the Norwegian Barcode of Life Initiative

Torbjørn Ekrem

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NorBOL (http://www.norbol.org) was formed in 2007 as a national network to 1) advance barcoding of Norwegian and Arctic biodiversity, 2) raise funding, 3) curate barcode reference material, 4) coordinate and initiate new barcoding projects, and 5) increase public awareness of DNA barcoding and barcoding results in Norway. NorBOL is a regional node within iBOL, with a particular responsibility for polar regions. The network is coordinated by the NTNU University Museum in Trondheim and connects 16 institutions, including all four major natural history museums as well as all major research institutes in Norway.

Despite strong support among research institutions, substantial external funding was only first obtained in 2012 through a grant from the Norwegian Biodiversity Information Centre (2012-2018). In 2014 a NOK 25.6M grant from the Research Council of Norway was secured to develop NorBOL into a distributed national research infrastructure on DNA barcoding. The external funding has progressed the establishment of a DNA barcode library of the Norwegian fauna, flora and fungi, and the Barcode of Life Data Systems database currently holds about 14000 DNA barcodes of more than 4400 species from Norway. The target for NorBOL is set to 20,000 species by 2018.

My presentation will focus on the organization of NorBOL and discuss important elements for building the network as well as obtaining funding. I will also show some actual examples of how studies connected to NorBOL has advanced our knowledge of Norwegian biodiversity.

Session II: DNA barcoding and Databases
15:00–15:30

DNA barcoding of Japanese chironomids

Natsuko Ito Kondo, Ryuhei Ueno, Kenzi Takamura

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Larvae of non-biting midges (Insecta: Diptera: Chironomidae) inhabits in various aquatic environments from brackish water to hot springs. They adapt to species-specific enrironments, and hence they are promising indicators for aquatic and ecotoxicological monitoring. Difficulty in morphological identification especially for larvae and adult females used to limit the utility as indicators, but DNA barcoding defuses the situation.

The DNA barcoding project of Japanese chironomids was launched on 2010. The objectives of the project are to provide the reliable databases of chironomids and promote their use for monitoring and researches on environmental studies. For the first step, about 100 common species within 1,500 species described in Japan would be disclose the species information with mitochondorial *COI* sequences. At present, 50 species are released on the website in Japanese, but the data will be internationally within 2014 and continue to be updated **(**http://www.nies.go.jp/yusurika/index.html**)**.

The progress in the barcoding project revealed the necessity of re-examination of species identification in some species. The necessity of consistency in species identification between countries and localities were revealed, too. We found the inconsistency of specie’s name between identical sequences submitted from different research groups. Some of these species can be a cryptic species. For promt and applied use of DNA barcodes in chironomids, we should carefuly discuss those cases together with thier morphology and ecology.

We would introduce our recent attempts of applying the DNA barcode of chironomids to environmental studies in reservoirs and also to the long-term monitoring of biodiversity in Lake Kasumigaura by using environmental DNA (eDNA).

Session II: DNA barcoding and Databases
15:30–16:00

DNA barcoding plant-insect interactions and activities of Japanese Barcode of Life Initiative (JBOLI)

Keiko Kishimoto-Yamada, Motomi Ito

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DNA barcoding gut-content analyses are an effective approach to constructing food webs of both aquatic and terrestrial species. We employed DNA barcdoing to reveal the trophic associations of adult leaf-chewing chrysomelid beetles in a Bornean rainforest[1]. Plant material ingested by the chrysomelid adults was retrieved from the insect bodies and a portion of the chloroplast *rbcL* sequence was then amplified from this material. The plants were identified at the family level using an existing reference database of chloroplast DNA. The study successfully identified the host plant families for eleven chrysomelid species. Our finding indicates that most of these chrysomelid species feed on multiple plant families across subdivisions or divisions. This contrasts with earlier estimates of host specificity among tropical insect herbivores. Our study also found more interaction types between plants and chryosmelid species compared with the results by direct observations[2].

In addition, we will introduce our activities of Japanese Barcode of Life Initiative (JBOLI), which is a project to provide information on DNA barcoding such as the latest news and protocols through the website (http://www.jboli.org), to host workshops and to support for activities relevant to DNA barcoding in Japan. At present, in collaboration with JBOLI, eleven DNA barcoding projects are in process.

References:

1. Kishimoto-Yamada et al. (2013) PLoS ONE 8 (9), e74426.
2. Kishimoto-Yamada and Itioka (2008) Biotropica, 40 (5), 600-606.

Session III: Biodiversity and Next Generation Sequencing
16:10–16:50

The expanding role of next generation sequencing in the research of coral-dinoflagellate symbioses

Todd C. LaJeunesse

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The application of molecular-genetic techniques has transformed our perceptions and expanded our understanding of a microbial dominated world. The more we study them the more we learn how microbes are critical to nearly every biological process in one way or another. Their common role in symbiotic interactions is a notable example. The persistence and growth of all coral reef ecosystems are dependent on the intimate symbioses between basal animals, corals, and microalgae, dinoflagellates in the genus *Symbiodinium*. Phylogenetic and population genetic data obtained over the past two decades have unveiled an unexpected diversity of these symbionts. This recognition has led to foundational advances in our understanding the ecology and evolution of these symbioses. With the rise next generation sequencing (NGS) and bioinformatics, we are poised to proceed further into the borderlands of scientific inquiry by, for example, identifying genes under selection during processes ecological specialization (i.e. speciation) or genes potentially important to the physiological change and adaptation of these symbioses to rapid climate change. In other research NGS may possibly aide in discovery the molecular basis of host-symbiont specificity and other immune-response pathways. The success of such endeavors will require further development of an experimental model system. While efforts to realize this have focused mainly on the finding a suitable animal host, the establishment of a corollary experimental system based on the symbiont has, until recently, been overlooked.

Session III: Biodiversity and Next Generation Sequencing
16:50–17:20

Dynamics of local adaptation from standing genetic variation in a wild *Arabidopsis* species

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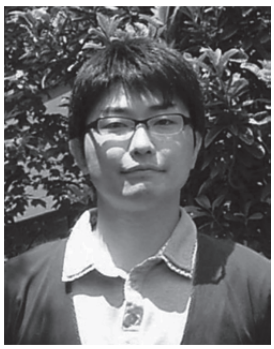

Wild populations often face drastic environmental changes, however, standing (pre-existing) genetic variants facilitate rapid adaptation to new selective pressures. The process of beneficial mutations to spread through the population is one of the most dynamic and fascinating phenomena in evolutionary biology, and at the same time highlights the importance of genetic diversity for adaptation. Moving beyond model organisms, the development of population genomic approaches has enabled to explore for genes underlying natural selections in non-model species. Through genome-wide SNP based population genomics, the present study explores the spatio-temporal dynamics of genes during local adaptation in *Arabidopsis halleri* subsp. *gemmifera*.

To observe the ‘spatial’ dynamics, two mountains were selected where similar highland ecotypes are found, presumed as a result of convergent evolution. By comparing the genomes from the bottom to the top of each mountain, genes that show allele frequency shift along the altitude were extracted. Many of these genes had functions related to various stresses, which are excellent candidates for the genetic basis of local altitudinal adaptation. Notably, some of the candidate genes were shared between both mountains, suggesting a common natural selection on genetic variants pre-existing before the divergence of the two mountains.

For the ‘temporal’ analysis, museum specimens of the two mountains that were collected at various time points (from 1904 to 2010) were used. From inter-time point genome comparisons, genes that alter their allele frequency across the time line were detected. Together with the results from the ‘spatial’ analysis, altitudinal allele frequency clines of some genes turned out to be shaped within a few decades. Although it remains necessary to determine the functions of the candidate genes, spatio-temporal genome comparison illustrate the rapid and dynamic process of genes during local adaptation.

Session III: Biodiversity and Next Generation Sequencing
17:20–17:50

Deciphering and prediction of transcriptome dynamics in a real world

Atsushi J. Nagano

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Recent advances in plant molecular biology have revealed large effects of the circadian clock, organism age, and environmental stimuli on transcriptomes under simple, controlled laboratory conditions. However, the factors that control transcriptomes under natural conditions are largely unknown. We have developed statistical models using extensive field transcriptome data and the corresponding meteorological data[1]. We named this approach as “field transcriptomics”. We showed that the transcriptome dynamics of rice leaves in a paddy field were mainly governed by ambient temperature and endogenous diurnal rhythms, as well as by plant age and solar radiation. We also found diurnal gates for environmental stimuli, detected associations between the thresholds for plant response to solar radiation and signal-to-noise ratios for day-length change, and predicted transcriptomes under given environmental conditions. Our models comprehensively describe transcriptome dynamics under complex field conditions and will help researchers to translate the vast molecular knowledge amassed in laboratories into solutions to problems in agricultural and natural environments. Now, we are trying to reveal seasonal physiological dynamics of perenial plant in its natural habitat by using field transcriptomics. We have finished RNA-Seq analysis of weekly samples for 2 years, from July 2011 to June 2013. We found very clear seasonal patterns in the transcriptome data of the hundreds samples, although the samples were collected from plants having variable genetic backgrounds and local environments. This data will help us to understand how plants robustly know seasons in nature.

Reference:

1. Nagano et al. (2012) Cell 151 (6), 1358-1369.

Poster presentations
11:50–13:50

P1	Takafumi Ishida	Establishment of a Mammalian Cell Repository - UT Frozen Bio Park-
P2	Takehito Kaneko	Sperm banking using freeze-drying in endangered animals
P3	Tomokazu Fukuda	Establishment of primary cell culture and immortalized cells from Loggerhead sea turtle, an ideal material for the genomic analysis with next generation sequencing
P4	Masanobu Kawachi	Role of microbial culture collection in progressing biodiversity and monitoring projects
P5	Ryuhei Ueno	DNA barcoding revealed some unknown species beneath the well-known species of the Chironomidae (Diptera)
P6	Shin-ichiro S. Matsuzaki	First record of exotic bagrid catfish *Tachysurus fulvidraco* in Lake Kasumigaura; their invasion, establishment and potential impacts
P7	Saki Harii	Challenge in mesophotic coral species identification: Can DNA barcoding help?
P8	Wataru Makino	DNA barcoding of Japanese freshwater calanoid copepods
P9	Kensuke Yoshimura	DNA barcoding on Japanese woody plants
P10	Yohei Shimura	Molecular phylogenetic diversity of Oscillatoriales maintained in MCC-NIES
P11	Kako Ohbayashi	The effect of severe Tsunami disturbances (Tohoku Earthquake) on genetic structure of a near-threatened tidal marsh plant *Carex rugulosa* in the Tohoku region, Japan
P12	Yayoi Takeuchi	Genetic diversity and reproductive success of a Bornean tropical tree, *Shorea laxa*, in a fragmented remnant forest used by local communities
P13	Masanori Tamaoki	Alteration of *Arabidopsis SLAC1* promoter and its association with natural variation in water loss resistance
P14	Hideyuki Ito	Analysis of genetic structure in three zebra species using microsatellite markers

P15	Yu Sato	Developing new microsatellite markers using next generation sequencer for endangered golden eagle
P16	Hitoshi Araki	eDNA for investigating unrevealed aquatic biodiversity
P17	Haruyo Yamaguchi	Picoeukaryotic diversity in the Northwestern Pacific Ocean based on amplicon sequencing of 18S rRNA gene
P18	Takafumi Kataoka	Community compositional analysis using 454 sequencing applying cryopreserved eukaryotic picopytoplankton originated from marine environments
P19	Frederic Sinniger	Environmental DNA survey of metazoan biodiversity: A case study in hydrothermal vent sediments
P20	Haruko Ando	Feeding ecology of the endangered red-headed wood pigeon *Columba janthina nitens* estimated by high throughput sequencing approach
P21	Keita Tsukahara	Fecal metagenomic analysis for identification of food habits and relation with accumulation of radioactive cesium of Large Japanese field mouse (*Apodemus speciosus*)
P22	Hikaru Saji	Gene response in rice plants treated with continuous fog influenced by pH, was similar to that treated with biotic stress
P23	Mieko Kono	Gene expression differences between lichens in hydrated and desiccated states

P1

Establishment of a Mammalian Cell Repository - UT Frozen Bio Park-

Takafumi Ishida[1], Tomoki Nishioka[1], Kazunari Matsudaira[1], Tsubasa Onitsuka[1], Kazutoshi Takami[2], Miya Ueda[3]

[1] Unit of Human Biology and Genetics, Graduate School of Science, University of Tokyo, Japan, [2] Osaka Municipal Tennoji Zoo, Japan, [3] Yokohama Zoological Gardens, Japan

Preservation of biological materials including endangered species should be our most important mission in the 21st Century. Over the last 30 years we have collected and preserved biological specimens namely mammals under the collaboration with zoos, gardens, parks, institutions and hunters. So far, more than 400 culture lineages from some 70 mammalian species have been preserved in liquid nitrogen. At the same time, we have ameliorated culture techniques to establish a novel cell immortalization protocol. We also demonstrated the use of human lymphocryptovirus (HHV-4; EBV) to be highly efficient to immortalize not only ape B-cells but those of the colobines, while the primate lymphotropic retrovirus has been shown to immortalize cells from a wide range of mammalian species. This primate oriented repository covers 33 primate species with 200 cell lines. The type of cells is namely fibroblastic or lymphoblastic. In this symposium, we present our achievement in the non-profit frozen repository and technical improvements.

P2

Sperm banking using freeze-drying in endangered animals

Takehito Kaneko[1], Hideyuki Ito[2, 3], Hidefusa Sakamoto[2, 3], Manabu Onuma[4], Miho Murayama[3]

[1] Institute of Laboratory Animals, Graduate School of Medicine, Kyoto University, Kyoto 606-8501, Japan, [2] Kyoto City Zoo, Kyoto 606-8333, Japan, [3] Wildlife Research Center of Kyoto University, Kyoto 606-8203, Japan, [4] National Institute for Environmental Studies, Ibaraki 305-8506, Japan

Sperm preservation is a useful tool for conservation of endangered animals. Freeze-drying sperm have been studied as new preservation method in various mammals as samples can be preserved at 4°C (refrigerator) or ambient temperature. We established the freeze-drying method that mouse and rat sperm could be preserved long term at 4°C after freeze-drying using a solution containing 10 mM Tris and 1 mM EDTA (TE buffer) (PLoS One 7, e35043, 2012, Cryobiology 64, 211-214,

2012). This study showed that the chimpanzee, giraffe and jaguar sperm could be maintained their fertility after freeze-dying using our protocol. Freeze-drying is the ultimate method by which sperm can be stored that neither required specialized cryoprotectants nor constant supply of liquid nitrogen. A further advantage is that freeze-dried sperm can be transported oversea at ambient temperature. Freeze-drying preservation protects strongly valuable gametes of endangered animals even in the event of unexpected accidents and disaster such as earthquakes and typhoons. Present, freeze-drying sperm has been applied as “Freeze-drying Zoo” for conservation of endangered animals (http://www.anim.med.kyoto-u.ac.jp/reproduction/home.aspx).

P3

Establishment of primary cell culture and immortalized cells from Loggerhead sea turtle, an ideal material for the genomic analysis with next generation sequencing

Tomokazu Fukuda[1], Kenichiro Donai[1], Takahiro Eitsuka[2], Masanori Kurita[3], Tomomi Saito[4], Hitoshi Okamoto[3], Kodzue Kinoshita[5], Masafumi Katayama[1], Makoto Soichi[3], Takafumi Uchida[1], Manabu Onuma[6], Hideko Sone[7], Miho Inoue-Murayama[5], Tohru Kiyono[8]

[1] Graduate School of Agricultural Science, Tohoku University, Sendai, Miyagi, Japan, [2] Faculty of Applied Life Sciences, Niigata University of Pharmacy and Applied Life Sciences, Niigata, Japan, [3] Port of Nagoya Public Aquarium, Nagoya, Japan, [4] Usa Marine Biological Institute, Kochi University, Tosa, Kochi, Japan, [5] Wildlife Research Center, Kyoto University, Kyoto, Japan, [6] Ecological Genetics Analysis Section, Center for Environmental Biology and Ecosystem, National Institute for Environmental Studies, Tsukuba, Ibaraki, Japan, [7] Environmental Exposure Research Section, Center for Environmental Risk Research, National Institute for Environmental Studies, Tsukuba, Ibaraki, Japan, [8] Division of Virology, National Cancer Center Research Institute, Tokyo, Japan

Background and Aim of the study: In this study, we tried to cultivate primary cells derived from Loggerhead sea turtle, which is one of the critically endangered animals. Furthermore, we tried to immortalize them with the intact karyotype and original nature maintained.

Materials and Method: We started the primary culture from the skin tissues of the Loggerhead sea turtles at 30 degrees centigrade. Proliferation rate of the cells was slower than those of most mammalian cells generally cultivated at 37 degrees centigrade.

Results and Discussion: The karyotype analysis of the Loggerhead sea turtle cells showed 2n=56 pattern, which is identical with Hawksbill, and Olive ridley. Furthermore, we are trying to

immortalize them by transducing the cell cycle regulators and activating telomerase. Immortalization of cells from endangered species including the Loggerhead sea turtle would enable us to share the cell lines worldwide, and they would be excellent research materials for the scientists.

P4

Role of microbial culture collection in progressing biodiversity and monitoring projects

Masanobu Kawachi
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Microbial Culture Collection at the NIES (NIES-Collection, http://mcc.nies.go.jp) was established as an “environmental study-oriented” culture collection in 1983. The collection maintains more than 3,000 strains, among which ca. 2,300 strains are available to the public. These strains include, in addition to model organisms, evolutionarily and ecologically important organisms. NIES-Collection is a public dedicated culture collection and have distributed strains for many purposes as well as received new strain depositions. Constant and smooth connection exists in between the algal research projects in NIES and the NIES-Collection. Bilateral benefits between project and culture collection will be introduced for several topics with highlight on biodiversity of hydrocarbon producing algae and monitoring studies targeting freshwater plankton and coral symbionts.

P5

DNA barcoding revealed some unknown species beneath the well-known species of the Chironomidae (Diptera)

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Larvae of Chironomidae (Diptera) are very important invertebrates in freshwater environments. But

their closely-similar morphologies, especially in larval forms, have often caused problematic identifications. Therefore, accurate species-identification measures with the assistance of molecular taxonomy is urgently needed. In this study, phylogenetic relationships among chironomid species have been investigated using DNA barcoding (sequences from a cytochrome *c* oxidase subunit I gene (*COI*)). The analysis mostly supports the conventional systematics based on morphology, although several inconsistencies appeared between morphological identificaton and DNA barcoding, even in the morphologically well-established species. For example, Japanese *Chironomus plumosus*, seemed to be a different taxa from the European *C. plumosus*. *C. nipponensis*, believed to adapt to a wide range of altitude from highland to lowland, seemed to be a mixture of two different species. In the studies of *C. kiiensis* and *Tanypus kraatzi*, similar phenomena are observed.

P6

First record of exotic bagrid catfish *Tachysurus fulvidraco* in Lake Kasumigaura; their invasion, establishment and potential impacts

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We collected eight specimens (28.2–170.2 mm SL) of exotic bagrid catfish from the Lake Kasumigaura, Ibaraki Prefecture, central Japan, during December 2008 and November 2011. We identified this species as *Tachysurus fulvidraco*, which is native to China and Korea, based on genetic and morphological analyses. We sequenced the mitochondrial control region (413 bp) and COI barcoding region (623 bp). Three juvenile specimens of this invasive species indicated successful reproductive activity in the lake system. The species is known to have similar morphological and food habits to channel catfish *Ictalurus punctatus*, which has also invaded Lake Kasumigaura, causing damage to the ecosystem and problems for local fisheries. The establishment and future habitat expansion of *T. fulvidraco* would also cause serious ecological and economic problems.

P7

Challenge in mesophotic coral species identification: Can DNA barcoding help?

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Okinawan coral reefs harbor some of the highest biodiversity found in Japan. Recently, anthropogenic activities as well as climate change are seriously threatening such ecosystems. To understand the resilience, recovery and adaptation potentials of Okinawan coral reefs, it is essential to estimate their biodiversity. Unfortunately, traditional taxonomy of scleractinians is based on skeletal structure requiring high expertise. In addition, molecular analyses showed that this morphological approach is not totally reliable as several unrelated groups present the same characteristics and morphology can change drastically with environmental factors. For all the above reasons, a DNA barcoding approach offers unique possibilities to develop a standardized evaluation of coral biodiversity.

Recently, healthy populations of corals locally extinct in the shallow surrounding waters were discovered at mesophotic depth (between 30 and 60 m depth). The Deep Reef Refugia Hypothesis (DRRH) states that some shallow coral species might find refuge in deeper areas where some physical stresses are less marked, especially temperature. The re-discovery of extinct corals suggests that in Okinawa, mesophotic coral ecosystems (MCEs) could indeed act as refugia.

In this study we used a combined approach of DNA barcoding and morphological examination in order to assess the coral diversity in local MCEs and understand the connection between deep and shallow communities. We sequenced mitochondrial and nuclear markers for over 300 coral specimens. In general the results were in good agreement with the rough morphology observed, however in a few situations, molecular analyses highlighted some extensive cryptic diversity and incongruences between nuclear and mitochondrial markers. Here we will present the results obtained and discuss on the potentials and pitfalls of the DNA barcoding applied to local coral reefs in Okinawa as well as our aim to develop a reference database for mesophotic corals in Japan.

P8

DNA barcoding of Japanese freshwater calanoid copepods

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Our previous study (Makino et al. 2013, Limnology 14: 269-282) has established a 28S ribosomal DNA regional sequence library (240 bp) of Japanese freshwater calanoid copepods (11 species), which is aimed to identify diapausing eggs in lake sediments. We have sequenced also the mitochondrial cytochrome *c* oxidase subunit 1 gene (mtCOI, 600-1100 bp) for the same 11 species. Here we integrate these nuclear and mitochondrial sequences in order to examine the taxonomic status of the 11 species. *Acanthodiaptomus pacificus* (Burkhardt) is judged as a cryptic species complex based on the degree of mtCOI sequence divergence among lineages that are distributed allopatrically (ca. 15-20% as K2P divergence) and reciprocal monophyly in nuclear and mitochondrial topologies. The rest 10 species are judged as “good species”, although divergence in mtCOI is relatively high (13%) in *Eurytemora affinis* (Poppe) and *Neutrodiaptomus formosus* (Kikuchi) compared with those in other 8 species (1-6%). We also compare our mtCOI sequences with those from freshwater calanoid copepods in neighboring countries.

P9

DNA barcoding on Japanese woody plants

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DNA barcoding in plants, Consortium for the Barcode of Life (CBOL) Plant Working group recommended a core-barcode consisting two plastid regions, rbcL partial sequence and matK partial sequence at 2009. We have been collecting herbarium specimens and DNA samples of Japanese woody plants about 7000 samples, 977 species. DNA sequences are analyzed on rbcL, matK and trnH-psbA as targets of DNA barcoding. The trnH-psbA intergenetic spacer region has the most variable information, and rbcL has the least. In contrast, the barcode region of rbcL is easy to amplify and sequence. The combination of rbcL + matK is proper backbone for the core-barcode dataset, but low discrimination power is far from perfect. It is necessary to use supplementary

barcode region.

P10

Molecular phylogenetic diversity of Oscillatoriales maintained in MCC-NIES

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Microbial culture collection at National Institute for Environmental Studies (MCC-NIES) maintains culture strains of cyanobacteria, eukaryotic microalgae, protozoa and endangered algae. In the culture collection, 713 cyanobacterial strains account for approximately 30% of all. Most cyanobacterial species have been described based on morphological features, and cyanobacterial strains in MCC-NIES have also been identified based on the morphology. However morphological identification needs high skills, and the general appearances (e.g. colony form, color and so on) may change during long-term sub-culturing. Nowadays, molecular phylogenetic analysis using 16S rDNA has become popular method for the classification in cyanobacteria. Oscillatoriales is an order of cyanobacteria characterized by non-branching filamentous form without heterocyst. According to the previous molecular phylogenetic works, it is already known that Oscillatoriales is not a monophyletic group. In this study, we targeted cyanobacterial strains classified as members of Oscillatoriales in MCC-NIES and re-identified those strains based on 16S rDNA sequences. Our results indicate high diversity of Oscillatoriales in MCC-NIES and several sequences obtained in this study displayed low similarities to any known cyanobacterial sequences, suggesting those novelty in taxonomy.

P11

The effect of severe Tsunami disturbances (Tohoku Earthquake) on genetic structure of a near-threatened tidal marsh plant *Carex rugulosa* in the Tohoku region, Japan

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Since the sandbars have declined due to river embankment and riverbed excavation, the numbers of organisms living in tidal marsh have also diminished. The tidal marsh halophyte, *Carex rugulosa*, was commonly observed along brackish sandbars in estuarine basins of Japan in past days. However, it has been designated as a near-threatened species on the Red Data list of Japan at present. We checked distribution status and analyzed genetic composition of populations in the western and the northeastern parts of Japan including the Tohoku region in 2008. We used SSR markers and clarified three genetically distinct clusters. Many coastal lines were destroyed by Tsunami caused by Tohoku Great Earthquake in 2011 in the Tohoku region. In 2013, we tried to search originally distributed populations and collected leaves of extant populations to analyze genetic composition using SSR markers. We discuss the effect of Tsunami disturbances on genetic structure of a tidal marsh plant in the Tohoku region.

P12

Genetic diversity and reproductive success of a Bornean tropical tree, *Shorea laxa*, in a fragmented remnant forest used by local communities

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Forest fragmentation affect tree reproductive and regeneration processes and cause low seed production, seedling survival or genetic diversity. We investigated whether the fragmented forest affects the reproductive success of a tropical tree, *Shorea laxa* (Dipterocarpaceae). First, we compared survivorship during the flower-to-seedling stages, seed fate and seedling mortality between a fragmented forest and a primary forest. Through microsatellite analysis using seeds, we examined the pollination process including the selfing rate, genetic diversity and pollen dispersal distances. As a result, we found no clear differences in tree survivorship. Although pollen dispersal was limited within the fragmented forest, a low selfing rate and high genetic diversity of the seed

array indicated effective pollination. In this study, we did not find a strong negative effect of forest fragmentation on the reproduction and regeneration of *S. laxa*, which indicates that remnant forest could play an important role on local genetic diversity conservation at least in a short term.

P13

Alteration of *Arabidopsis SLAC1* promoter and its association with natural variation in water loss resistance

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Water availability and temperature strongly limit the natural distribution of terrestrial plant species. Comparing drought-adapted and non-adapted species would be a desirable approach to studying plant adaptation to local water conditions. *Arabidopsis thaliana* is distributed widely in the Northern Hemisphere and has experienced a wide range of climatic conditions and selective pressure for thousands of generations. Here, we investigated water loss resistance in 41 *Arabidopsis* accessions, and found that Columbia (Col-0) and Wassilewskija (Ws-2) accessions differ in the ability to resist water loss stress. Since it was presumably due to genetic differences, QTL analysis was performed to identify the applicable trait loci. The result indicated a locus on chromosome 1. Surveying in the locus, we extrapolated that the *SLAC1* gene, which is associated with stomatal closure. Comparison of their sequences found that there was no significant difference between the SLAC1 proteins but was a significant change in *SLAC1* promoter region. We determined the *SLAC1* promoter activity by histochemical GUS staining for *SLAC1* expression site and by quantitative PCR analysis for *SLAC1* transcript level. Although the *GUS* expressed at guard cells in both accessions, the transcript level of *SLAC1* was dominantly higher in guard cells of Col-0. The results indicated that the *SLAC1* promoter was a key player in determining the difference in stomatal control of water loss between Col-0 and Ws-2.

P14

Analysis of genetic structure in three zebra species using microsatellite markers

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Zebras are members of the horse family (Equidae) in Africa. There are three species of zebras: the plains zebra *E. quagga*, the Grevy's zebra *E. grevyi* and the mountain zebra *E.zebra*. The Grevy's zebra and the mountain zebra are endangered, and hybridization has been recorded between the Grevy's zebra and the plains zebra, so it is necessary to provide genetic information within/between species for conservation. We developed 28 microsatellite (MS) markers for Grevy's zebra and tried cross-amplification in two other zebras. For Grevy's zebra, the number of alleles ranged from 2-9, and the observed and expected heterozygosities were 0.019–0.885 and 0.019–0.782, respectively. Cumulative probability of identity was low (2.3×10^{-14}), indicating that these markers are enough for individual discrimination. All markers were successfully amplified in two other zebra species, and almost all markers were polymorphic. These markers are useful for clarifying genetic structure of zebra species, and for identification species and subspecies. We think that MS markers developed in this study will be useful for conservation of endangered zebra species in wild and captivity.

P15

Developing new microsatellite markers using next generation sequencer for endangered golden eagle

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Golden eagle (*Aquila chrysaetos japonica*) is one of the most endangered species in Japan. There are many ecological studies, but only a few genetic studies. Developing species specific new genetic markers such as microsatellite markers is important to genetic study, because markers are necessary for more precise study such as individual identification, a parent-child relationship or kinship. So, we try to establish new microsatellite markers using next generation sequencer.

DNA was extracted from a blood sample of a female golden eagle bred in Akita Omoriyama Zoo. Sequencing was conducted using next generation sequencer ion torrent (Life technologies). We obtained 5,222,991 reads. Their mean length was 269bp and total bases were 1.4GB. 2,424 fragments were selected for following condition; more than seven repeats of two to four base unit sequence by Msatcommander. We designed microsatellite markers with PRIMER3. Then we are going to characterize these markers for number of alleles, observed and expected heterozygosities (*Ho* and *He*), and probability of identity (*P-id*) using DNA samples extracted from feathers, pellets and egg membrane of wild golden eagles.

P16

eDNA for investigating unrevealed aquatic biodiversity

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Environmental DNA (eDNA) is a recently developed, metagenomic approach to investigate higher organisms in natural environment. The eDNA technique, in combination with the next-generation sequencing, is rapidly developping worldwide, although there still are many technical issues around it. We introduce our plans for research projects utilizing this technique to understand unrevealed fish biodiversity in open water. Because it requires only small volume of environmental water sample, successful development of such a technique has a huge potential for wider range of applications in aquatic researches. We will discuss the potential, pros and cons of the eDNA technique with the next-generation sequencing technology.

P17

Picoeukaryotic diversity in the Northwestern Pacific Ocean based on amplicon sequencing of 18S rRNA gene

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Recent several genetic analyses have revealed unexpected diversity of picoeukayotes in marine environments. However, limited number of species has been described so far, and many sequences categorized as uncultured and undescribed species are accumulating in gene banks. In addition, genetic diversity of the picoeukarotes in the open sea around Japan is still remained unclearly. The present study focused on genetic diversity of marine picoeukaryotes in two regions of the Northwestern Pacific Ocean. We used seawater samples taken in February 2011 and July 2013 for amplicon analyses of 18S rRNA gene. Crypreservation of the cells and flowcytometry are applied to the sample preparations. 454 sequence analyses showed seasonal differences from the two regions. The detailed survey of picoeukaryotic biodiversity would provide basic information for future monitoring study in the open sea around Japan.

P18

Community compositional analysis using 454 sequencing applying cryopreserved eukaryotic picopytoplankton originated from marine environments

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Eukaryotic picophytoplankton is ecologically and biogeochemically significant component in the marine microbial food web. Recently study about diversity of marine microbes has been accelerated using molecular techniques, but knowledge of eukaryotic phytoplankton diversity are fragmented. Especially on eukaryotic picophytoplankton (less than 3 μm), basic information about diversity is still limited because of (i) lacking the 18S rDNA data in public database and (ii) fragile trait of the cell preventing sample collection. In this study, we applied cryopreserving technique to marine environmental specimens obtained from 2 sites in Sendai bay for the purpose of preserving whole natural biodiversity. Then 2,800 picophytoplankton cells were selectively collected using flowcytometry equipped with a cell-sorter to analyze 18S rDNA composition using 454 amplicon analysis. From more than 10,000 reads, 28–46 operational-taxonomic-units (OTUs: 95% cut-off) were constructed in each sample. A cluster analysis showed that OTUs compositions were different between cryopreserved and non-cryopreserved treatments in both sites. Of the 94 OTUs, 11 OTUs

were remarkably detected in non-cryopreserved treatments, and the other 10 OTUs were done in cryopreserved treatment, respectively. Those results showed that sample preservation process would affect on the 18S rDNA amplicon analysis.

P19

Environmental DNA survey of metazoan biodiversity: A case study in hydrothermal vent sediments

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The study of biodiversity is now strongly limited by the availability and expertise of specialist taxonomists for many metazoan taxonomic groups. In addition to this impediment, deep-sea biodiversity is also challenged by the difficulty to access the targeted ecosystems. Development of high-throughput DNA sequencing opened new perspectives in the study and exploration of marine biodiversity. The survey of genetic diversity in an ecosystem using environmental DNA (total DNA directly extracted from an environmental sample) showed promising perspectives to explore the taxonomic diversity in the remote environments of the deep sea. Here we will discuss on the potentials and pitfalls of this approach in a deep-sea perspective. We will present our results obtained using high throughput sequencing of sediments collected in hydrothermal vents and other deep-sea environments between 1000 m – 9000 m depth. Our data allowed us to estimate benthic diversity in different hydrothermal conditions and to compare with various non-hydrothermal deep-sea environments. In addition, this approach also allowed us to detect for the first time, potential still unknown vent-endemic organisms. Such discovery illustrates very well the contribution of molecular methods to traditional biodiversity studies. Based on those results we will explore the future challenges toward using environmental DNA for marine biodiversity exploration and environmental assessment.

P20

Feeding ecology of the endangered red-headed wood pigeon *Columba janthina nitens* estimated by high throughput sequencing approach

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We studied feeding ecology of critically endangered red-headed wood pigeon *Columba janthina nitens*, endemic subspecies to a highly remote and disturbed oceanic island chain: the Ogasawara Islands. Analysis based on high throughput sequencing (HTS) was carried out for 628 fecal samples collected for two years in two island habitat: Chichijima and Hahajima. Food resource availability and nutrient composition of major fruits found in the two islands were also estimated. The results of HTS diet analysis detected 122 food plant taxa and showed clear seasonal and inter-island variation of pigeons' diet. Although the results of model selection indicated pigeons' preference of lipid rich fruits, diet composition and diet width were flexibly changed according to food resource availability. This flexibility of food selection may indicate the foraging strategy of the red-headed wood pigeon in isolated island habitat with poor food resources. Pigeons also consumed introduced plants temporary in high frequency, which may complement the lack of preferable native food resources. Degree of pigeons' dependence on introduced plants seems to differ between the two island habitats, thus different impact of introduced plants eradication on pigeons foraging condition in each island should be considered.

P21

Fecal metagenomic analysis for identification of food habits and relation with accumulation of radioactive cesium of Large Japanese field mouse (*Apodemus speciosus*)

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Emission of radioactive Cs (Cs-134 and Cs-137) at the Fukushima Daiichi nuclear disaster on March 2011 might exert some influence for wildlife and its habitat by γ-ray. In recent study,

accumulation of Large Japanese field mice (*Apodemus speciosus*) showed considerable individual variability. We researched and analyzed whether the accumulation of radioactive Cs of *A. speciosus* correlated with these food habits or not at the affected area, by metagenomic analysis using next-generation sequencer. 39 of *A. speciosus* were captured at high-dose area in Fukushima prefecture by using Sherman Trap, and these feces were corrected from intestines. Genomic DNAs extracted from feces were used for NGS analysis of food habits by DNA barcoding using ribulose-1,5-bisphosphate carboxylase/oxygenase gene large subunit (*rbcL*, for land plants) and mitochondrial cytochrome c oxidase gene subunit I (*COI*, for metazoan invertebrates). The food habits were estimated by these sequenced data, and investigated what species were correlated with the accumulation of radioactive Cs in *A. speciosus*.

P22

Gene response in rice plants treated with continuous fog influenced by pH, was similar to that treated with biotic stress

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Throughout Asia, including Japan, rice plants are cultivated in a wide range of areas from lowlands to highlands and are frequently exposed to fog, including acid fog. Some physiological studies have shown that acid fog can be a stress factor for plants. We analyzed the gene expression profiles of rice plants treated with artificially prepared simulated acid fog (SiAF) or simulated neutral fog (SiNF) for 1 or 7 days.

Microarray analysis results suggested that both the SiAF and the SiNF treatments induced the expression of genes involved in the defense and stress responses in rice plants although the influence of SiAF was more remarkable than SiNF. The comparison of gene expression profiles among plants treated with different stress factors revealed that both SiAF and SiNF treatments have similar effects to biotic stresses and ozone stress, suggesting that these fog treatments may result in oxidative stress.

P23

Gene expression differences between lichens in hydrated and desiccated states

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Lichens are symbiotic organisms consist of fungi and photosynthetic partners such as algae or cyanobacteria. They are adapted to environment with various water availability including extreme environments such as polar regions and deserts. It is known that metabolisms of lichens are switched on and off according to the water content of lichen thalli. Yet genetic mechanisms controlling their metabolisms remain unknown. In this study we incubated field collected *Usnea bismolliuscula* and *U. hakonensis* under 18°C, white fluorescent light ($50\mu mol\ m^{-2}s^{-1}$) in either desiccated or hydrated conditions. After 2 hours of incubation, mRNA was extracted from each sample and gene expressions were compared between the two conditions. As a result, 281 / 394 genes and 104 / 31 genes were identified as specifically expressed genes in desiccated and hydrated *U. bismolliuscula* / *U. hakonensis* respectively. Gene ontology (GO) analysis indicated the transcription of sugar transporters in the desiccated condition. However, most of GO categories related to the basic metabolism were common between the two conditions, implying some of metabolic differences between hydrated and desiccated lichens are not controlled at transcription level.

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