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Symposium Paper

The Application of Genetics in Balancing the Conservation and Utilisation of Biodiversity in Multi-Use Environments

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Biodiversity conservation and in changing environments

Environmental change is a significant challenge worldwide for biodiversity conservation and poses serious consequences for the management of plant communities. At the same time, our well-being depends on making use of diverse ecosystems and the plants within them so management plans must recognise the pressures imposed by multiple users and promote sustainable use of natural resources.

Habitat fragmentation and climate change are the two global drivers of current biodiversity loss and ecosystem change (Breed et al. 2013). To ensure that plant communities and species have the ability to respond to change, it is essential to maintain a balance between the ecological and genetic requirements of species and the modification of natural systems due to human use. We aim to mitigate the detrimental effects of human activity on natural ecosystems while acknowledging that environmental change has been a constant evolutionary force (Moritz 2002). Understanding the patterns of genetic diversity within species and across landscapes can assist in establishing conservation policy (Laity et al. 2015), prioritising conservation targets (Moritz & Potter 2013) and developing practices for sustainable use (Dickson & Cooney 2005).

Most plant species are unlikely to have the capacity to track climate changes by adjusting their geographic range because of dispersal limitations (Cunze et al. 2013). A mismatch between the rate of contemporary climate change and habitat loss, and the capacity of species to respond (Cunze et al. 2013; Jump & Peñuelas 2005), may precipitate a loss of biodiversity and a demise in ecosystem properties and processes (Malcolm et al. 2002). Human-assisted migration (Ste-Marie et al. 2011) and restoration based on matching genetics to predicted climates (Weeks et al. 2011) have been suggested as potential tools to maintain population and species viability.

The Australian flora

The Australian flora, estimated at more than 20,000 angiosperm species, is large, diverse and highly endemic. It is also considered very vulnerable to climate change (Preston & Jones 2006). A dramatic increase in environmental stressors is anticipated in coming decades (Dunlop et al. 2012). It is predicted that a majority of the Australian land mass, including south-eastern Australia, will experience climatic changes that result in significant environmental stress for organisms that are adapted to current conditions (Dunlop et al. 2012). Annual mean temperature anomalies in Australia since 1910 show a change from colder to warmer anomalies with the moving ten year average becoming positive from 1980 (Fig. 1). Based on the projections of the Australian Bureau of Meteorology, using data from 1970 onwards, most regions are expected to receive less rainfall (Fig. 2) and experience higher temperatures (Fig. 3).

Worldwide, there is an increased likelihood of extinction for many taxa (Groom et al. 2006). In Australia, 1265 plant species are considered to be critically endangered, endangered or vulnerable and 39 species have become extinct in recent decades (http://www.environment.gov.au/, accessed 14-9-2015). Many extant Australian plant species are likely to have experienced and responded to past climate change. However, the timescale has changed and the majority of Australian landscapes in which they exist have undergone the most dramatic changes in the last 200 years (Nix 1981). Farming, forestry and urbanisation have caused rapid and extensive human-mediated modification to vegetation systems and loss of habitat for many species. The disjunct distributions of many species most likely indicate that previously suitable habitat has been lost rather than long-distance dispersal and exploitation of new habitat. As many Australian species are restricted in distribution, regional extinction is likely to mean global extinc-

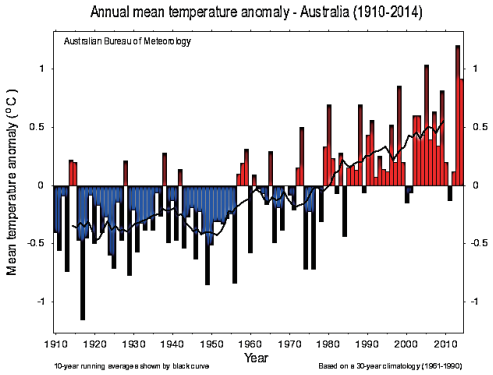

Fig. 1 Temperature anomalies across Australia based on 10 year moving average from 1910 (image from www.bom.gov.au).

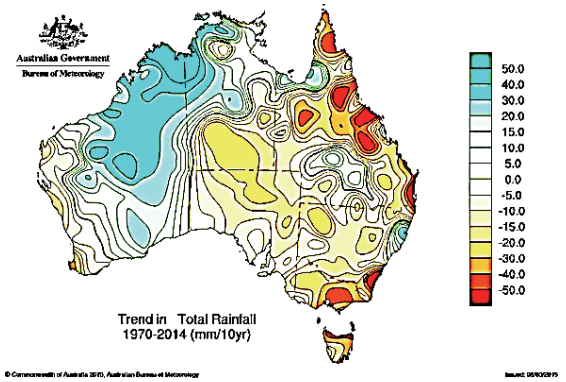

Fig. 2 Reduced rainfall over most of Australia since 1970 (image from www.bom.gov.au)..

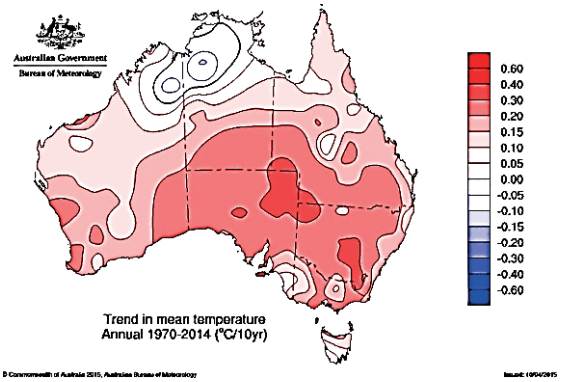

Fig. 3 Increased temperatures over most of Australia since 1970 (image from www.bom.gov.au).

tion.

Using genetic information for conservation

Conservation genetics uses the principles of population genetics and conservation biology to enhance the long-term viability of species and ecological systems. Conservation strategies must offset threats to species persistence to maintain species diversity and genetic diversity (Thuiller et al. 2008). There is a difference between the use of genetics for conservation (Broadhurst & Young 2007) and its application, for example, to the development of commercial crop lineages that are often limited in genetic diversity (Mohammadi & Prasanna 2003). Biodiversity conservation is reliant on dynamic evolutionary processes that enable adaptation in response to change (Sgrò et al. 2011) whether natural or human-mediated whereas vegetative propagation of a single genotype may be required to ensure a genetically uniform lineage that displays particular desirable traits.

Habitat fragmentation contributes to species decline and extinction because it can lead to small, isolated populations that are ultimately more susceptible to demographic fluctuations and the loss of genetic variation (Frankham 2005; Lienert 2004; Young & Augsberger 1991). The effects are not restricted to rare species; common plants may be equally or even more susceptible to the population genetic consequences of fragmentation depending on previous history of inbreeding and outcrossing (Honnay & Jacquemyn 2007).

The maintenance of evolutionary processes remains one of the major goals for the management of genetic resources (Sgrò et al. 2011). It is dependent on understanding the genetic consequences of habitat fragmentation and changing climate on populations (Hoffmann & Sgrò 2011; Young et al. 1996) because the capacity of ecosystems to respond to environmental changes is dictated by the ecological and genetic constraints on evolutionary processes (Stein et al. 2013). Genetic decline and demographic factors may affect the adaptive capacity of populations (Willi & Hoffmann 2009; Willi et al. 2006) but manipulating genetic diversity has the potential to enhance resis-

tance to stress (Pauls et al. 2013).

Genetic studies are particularly useful for managing species that reproduce vegetatively, where taxonomic uncertainty may affect the recognition of conservation targets, where divergent genetic lineages may represent different evolutionarily significant units (Moritz 1994) or management units (Palsboll et al. 2007) and for the management of genetic resources under predicted environmental change (Pressey et al. 2007; Thuiller et al. 2008; Weeks et al. 2011). The complexity of factors that affect ecological and evolutionary processes limits the applicability of generalisations to specific cases yet it is not possible to assess the conservation requirements of all species (Frankham 2010). Thus we must rely on more generally applicable guidelines such as the strategies detailed by Sgrò et al. (2011) designed to build evolutionary resilience into biodiversity conservation.

Species' persistence may be facilitated by managing for predicted ecological changes rather than existing or historical conditions (Seastedt et al. 2008; Weeks et al. 2011). This idea has led to recommendations for seed sourcing and composite provenancing (Broadhurst et al. 2008), where seed from a range of environments is combined for habitat restoration, to take into account the need to mitigate the effects of changing climate (Breed et al. 2013; Prober et al. 2015).

The use of a range of genetic techniques such as DNA sequence variation and the genotyping of individuals enables us to identify maternal lineages, to differentiate between individuals and clone mates and to accurately measure differences in genetic variation between individuals, populations and species. In conjunction with field-based information, better understanding of the patterns of diversity is leading to integrated management plans that try to balance the multiple pressures imposed by our use of resources with the essential requirements for biodiversity conservation.

Case studies

The following examples from south-eastern Australia show the application of genetic techniques to three different conservation issues:

1. Conserving clonal species

Population genetic analysis of two *Grevillea* species (Proteaceae) from the same informal taxonomic group of holly-leafed grevilleas has shown that both species are extensively clonal and sterile. Despite thousands of stems, *G. renwickiana* occurs in only a few populations in New South Wales and consists of eight clonal lineages (James & McDougall 2014) and *G. infecunda* (Fig. 4) occurs in only a few populations on the southern coast of Victoria and consists of 38 clonal lineages ((Kimpton et al. 2002), James, unpublished data).

Another rare plant, *Olearia passerinoides* subsp. *glutescens* (Asteraceae), was recently discovered in Victoria, hundreds of kilometres from known locations in South Australia. Genetic analysis has revealed that the five Victorian populations, all found within 10 km of each other, contain only 8 clones (Fig. 5) but South Australian populations have higher levels of genotypic diversity suggesting that they reproduce mainly from seed (Suyama et al, unpublished

Fig. 4 Holly-leafed *Grevillea infecunda*. This sterile species contains fewer than 50 clonal lineages despite the presence of several thousand stems. Image: E.James

Fig. 5 A population of *Olearia passerinoides* subsp. *passerinoides* (individuals marked with pink flags). Genetic analysis showed that the plants here belong to a single clone and there are only 8 clones in Victoria. (image: Karly Learmonth)

data).

Knowledge of the spatial distribution, fecundity and extent of clonality in these species allows them to be managed to ensure continued vegetative reproduction.

2. Threatened plants species of the Victorian Volcanic Plain

Native temperate grasslands are the most threatened ecosystems in Australia (Williams et al. 2015). The Victorian Volcanic Plain in south-eastern Australia has been converted to a highly productive agricultural region at the expense of native grassland communities. As a result, naturally occurring habitat is highly fragmented and occupies less than 1% of the 2.3 million hectares comprising the VVP (Williams et al. 2015) and the once extensive grasslands exist mostly as small, isolated remnants.

Patterns of genetic variation have been collected for 5 species, *Pimelea spinescens* (Thymeleaceae) (James & Jordan 2014) (Fig. 6), *Diuris basaltica* and relatives (Orchidaceae) (Ahrens et al. unpublished data), *Comesperma polygaloides* (Polygalaceae) (Ahrens & James 2016), *Senecio macrocarpus* (Asteraceae) (Ahrens & James 2015) and *Ptilotus microcephalus* (Amaranthaceae) (Ahrens & James 2016). All species showed a much lower level of population genetic structure than expected despite significant habitat destruction and small population size.

The conclusion reached from these studies is that, historically, the open plains of the VVP facilitated gene flow within the VVP and there has not been a significant decline in genetic diversity, in part because many of the plants are long-lived. The general lack of population structure does not reflect the recent habitat fragmentation of the VVP but instead reflects historical levels of diversity and gene flow and suggests a common factor that has influenced patterns of genetic diversity more generally across the VVP. The importance of these findings is that germplasm from throughout the VVP can be used for restoration purposes. The information will assist in managing the genetic diversity of each species to facilitate adaptive responses to environmental change.

Fig. 6 Protecting Pimelea spinescens, a threatened species of the grasslands of the Victorian Volcanic Plain, grows in remnant vegetation in a mosaic of agricultural land. Signs remind landowners and the general public that we value this plant. (Image: courtesy of the Spiny Riceflower Recovery Team)

3. Wetlands of the Gippsland Lakes

The Gippsland Lakes in south-eastern Australia is an extensive Ramsar-listed wetland system of > 60,000 ha. It supports commercial fishing, hunting, tourism and agricultural pursuits. It has experienced chronic salinisation since the late 19th century following the construction of a permanent channel to the sea (Fig. 7) to improve boat access (Boon et al. 2015). The effects were exacerbated in the mid-late 20th century when fresh water from inflowing rivers was increasingly regulated and extracted.

Changes to hydrology and the competing demands from users of the land and wetlands have led to substantial ecological impacts on the Lakes' ecosystem including changes in the distribution of *Melaleuca ericifolia* (Myrtaceae) and *Phragmites australis* (Poaceae) (Fig. 8). Both species establish from seed but clonal growth can be substantial. Lowered water levels allowed the establishment of *M. ericifolia* which became dominant with extensive clonality and replaced much of the *P. australis* (Fig. 9). Little genetic structure was observed in *P. australis* and was not correlated with salinity (Hurry et al. 2013; James et al. 2013). A recent transcriptome study of *P. australis* has identified differences in gene expression between plants sourced from high and low salinity sites when exposed to fresh water and high salt levels (Holmes et al. 2016). Evidence of adaptation to salinity may provide a basis for selecting germplasm for restoration.

Conclusions

Genetic studies have enabled significant advances in our understanding of populations, species, habitats and their conservation requirements. Studies ranging from rare species to widespread species and across

Fig. 7 A permanent opening of the Gippsland Lakes to the ocean was constructed in 1889. Salinity levels have risen since then and lakes have become increasingly salinised. Image: E. James

Fig. 8 A wetland in the Gippsland Lakes dominated by Common Reed, *Phragmites australis*. An individual plant of *Melaleuca ericifolia* is visible in the centre of the image. Image: E. James

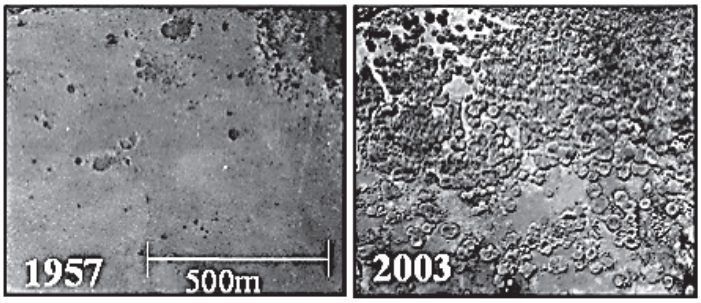

Fig. 9 The relative abundance of *Melaleuca ericifolia* and *Phragmites australis* changes with management. In 1957, *M. ericifolia*, visible as small dark areas, was uncommon and *P. australis* occupied most of the area. By 2003, large clones of *M. ericifolia* dominate. (Image modified from Robinson et al. (2012)).

different ecological niches are helping to build up a picture of the variability in patterns of genetic diversity and an understanding of the processes that have led to them. This will assist us balance our use of natural resources with the need to retain the evolutionary processes necessary for adaptation to the pressures of environmental change at all levels of biodiversity.

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Conservation Activities on Korean Rare and Endemic Plants —with a Special Reference to the Korea National Arboretum

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Abstract

The current status of conservation activities on the Korean rare and endemic species was reviewed with a special reference to the Korea National Arboretum (KNA). In the Korean peninsula, there are 4,172 plant taxa including 360 endemics and 571 rare plants categorized at the national level by the IUCN criteria: EW 4, CR 112, EN 199, LC 70, and DD 112. The KNA has established various *in-* and *ex-situ* conservation programs in practice for GSPC goals since 2010. In order to improve the conservation activities, the KNA has managed specialized research projects as well as networking programs with other local governmental arboreta and NGOs in Korea. The main purposes of the research projects are to (1) survey, update, and evaluate the conservation and genetic status of Korean populations of rare endemic plants; (2) formulate an urgent conservation strategy; and (3) monitor the endangered populations based on the latest and accurate ecological and biological information. For the *ex-situ* conservation, the KNA has secured and propagated seeds and living collections of rare and endemic plants, and as a result, the KNA conserves about 70% of Korean rare plants as living collection and propagules. A total of nine conservation fences have been installed and monitored by the KNA as part of *in-situ* conservation. In addition, the KNA has carried out a re-introduction program for rare plants such as epiphytic orchids in natural habitats by developing propagation techniques with accurate genetic tags. The KNA also promotes training and international cooperation programs for the *in-* and *ex-situ* conservation activities for the East Asian biodiversity.

Introduction

Brief history and current status of rare plant conservation in Korea (adopted form Korea Forest Service 2008)

The beginning of history of rare plant conservation in Korea began after the Korean War, when UN came to Korea and UNESCO under UN started its operation. The history of botany in Korea started with the introduction of main plant distribution by Western and Russian scholars in early 19th century and then was researched by Japanese scholars after the Japanese annexation of Korea. During tumultuous period after the independence in 1945 and Korean War, the plant research in Korea was performed locally by a few Western scholars. UNESCO played a central role in the research and its Korean partner was the Korea Association of Conservation for Nature (KACN). KACN was founded in December, 1963 as an academic inspection committee for natural resource conservation It was the only academic NGO in Korea back then. The members of KACN consisted of mostly scholars in biology and forestry. Not only scholars in botany, but also scholars in various field such as wild animals, insects and fungus participated in the organization. Important regional research results were presented through the organization's publication Nature Conservation Magazine, the only available journal related to biology during the period.

Due to rapid industrialization in Korea, numerous habitats for important plants were threatened and destroyed. Corresponding to these issues, biologists publicized conservation reports for plant habitats, which needed to be distinguished as protected species (Prof. Deok-bong Lee: the first two selected species – *Cypripedium japonicum*, *Berchemia berchemiaefolia*). Initiated by Deok-bong Lee who designated *Cypripedium japonicum*, *Berchemia berchemiaefolia* as protected species (1969), the status of protected

rare plants included 106 species presented by Mankyu Park (1975), 118 species by Young-no Lee (1981), and 79 species by Tchang-bok Lee (1987).

As Korea has become a more developed country, strategy to manage biodiversity and standardization of national biology management in policy are demanded. Since there are clear differences in the responsibilityies of central government, local government, educational institution, and NGO, the role of each organization in the efficient management for national biology will occur. In this case, it is recommended that for rare plants by criteria central government should research, conserve and monitor critically endangered species and endangered species, and for vulnerable and least concern species local government should take the lead in those conservation activities. For data deficient species identification of habitat and criteria review should be proceeded as a next step.

Purpose of This Research

4,172 plant taxa have been known in the Korean peninsula, including 360 endemics and 571 rare plants categorized at the national level by the IUCN criteria: EW 4, CR 112, EN 199, LC 70, and DD 112 (Korea Forest Service 2008). This research was carried out to generally understand the conservation activities for Korean rare and endemic plants in recent years with a special reference to the Korea National Arboretum (KNA). Most of the results were based on research papers and reports (especially Son 2015) about conservation activities or programs of the KNA.

Results and Discussion

The KNA has established various *in-* and *ex-situ* conservation programs in practice for GSPC (Global Strategy for Plant Conservation) goals since 2010 (Son 2015). In order to improve the conservation activities, the KNA has managed specialized research projects as well as networking programs with other local governmental botanic gardens and NGOs in Korea. The KNA started an important project titled "Construction of infrastructure for conservation of rare and endemic plants in Korea" in 2010 with a 10 years plan. The main purposes of the research projects are to (1) survey, update, and evaluate the conservation and genetic status of Korean populations of rare endemic plants; (2) formulate an urgent conservation strategy; and (3) monitor the endangered populations based on the latest and accurate ecological and biological information. About 24 research teams from universities, national, public, and private arboretum (or botanical gardens), research institutes, NGOs, etc, participate in this project. An outline of the project is presented in Fig. 1. Some remarkable case results from the research are as follows:

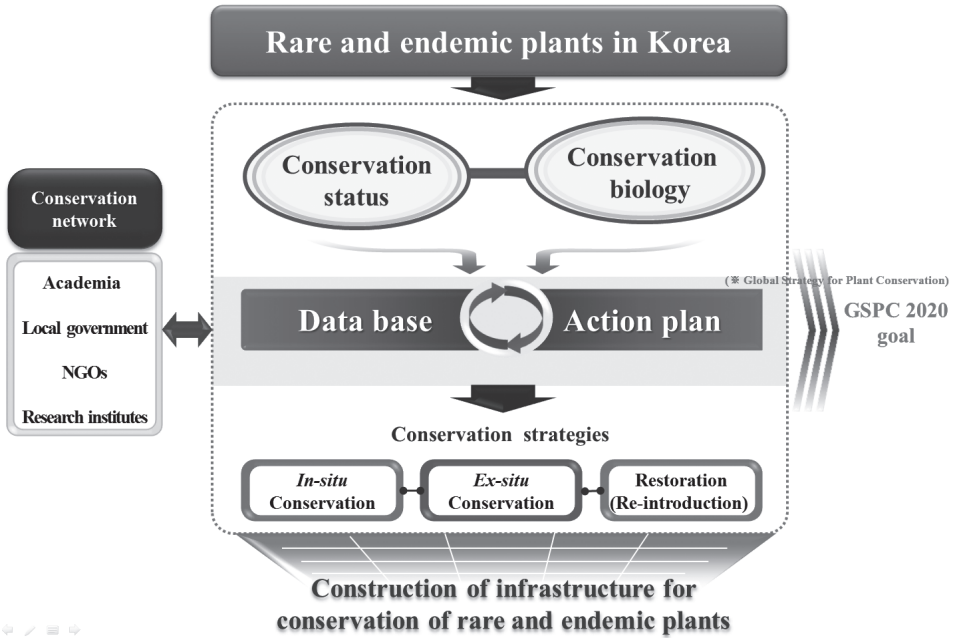

Fig. 1 An outline of the KNA project titled "Construction of infrastructure for conservation of rare and endemic plants in Korea".

1. Phytogeography on rare and endemic species (with two case studies)

***Scrophularia takesimensis* Nakai (Choi et al. 2012):** *S. takesimensis* Nakai is a critically endangered plant species endemic to Ulleung Island, Korea. The researchers provide updated information on the distribution and conservation status of this species. They located 39 subpopulations and counted a total of 443 individuals, including some reintroduced. Observations of dried and broken branches, with fruits, of *S. takesimensis* along the coast may indicate dispersal by sea. The construction of coastal roads is the main threat to the species. To conserve this species more effectively, the researchers recommend that: (1) the two habitats identified as a priority for conservation should be afforded special protection, (2) habitats to the seaward side of coastal roads are more suitable than the habitat on the landward side for in-situ conservation, and (3) the presently known subpopulations require continuous protection and monitoring.

***Quercus myrsinifolia* Blume (Lee et al. 2014):** Most evergreen *Quercus* species are typical, dominant members of Korean evergreen forests. However, little is known about the distribution status of *Q. myrsinifolia* Blume there. To enhance our knowledge about their natural range in Korea, the researchers conducted field surveys based on specimen records and an extensive literature search. They also determined their exact number as a first step in planning their conservation. The results indicated that these trees are strictly limited to Jin Island, and 169 mature individuals were the maximum number and occurred in only three subpopulations on that island. Previous misidentifications and perhaps mislabeled locations for plant specimens were the main reasons for the earlier confusion about distribution. The researchers believe that these results can provide us guidance required for making specific recommendations for management interventions. These discoveries also demonstrate the value in having reliable information about plant specimens in general. They also speculated about what makes this species particularly vulnerable to local extinction.

2. Ex-situ conservation

For the *ex-situ* conservation, the KNA has collected and propagated seeds and living collections of rare and endemic plants, and as a result, the KNA conserves about 70% of Korean rare plants as living collection and propagules (Son 2015). Main facilities of ex-situ conservation in KNA are seed bank, propagation center (greenhouse, bed, tissue culture room), and conservation gardens. The nine local governmental botanical gardens have established the conservation garden for the duplicated conservation of living collections of KNA. In particular, they try to manage the *ex-situ* conserved individuals with accurate genetic tags as proposed with a case study of *Euchresta japonica* Hook. f. ex Regel (Fig. 2; Choi et al. 2013).

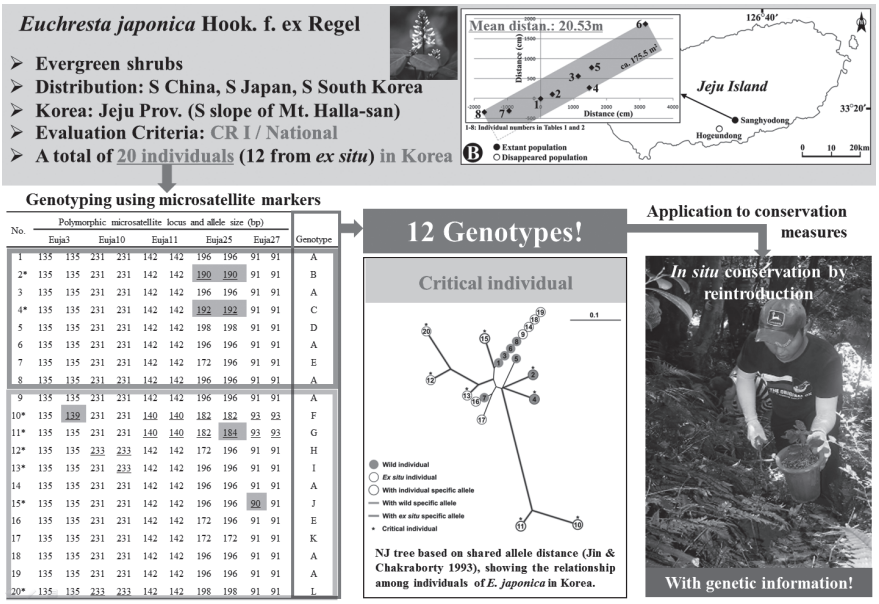

Fig. 2 The result of a case study (with *Euchresta japonica*) about the importance of genetic information in conservation measures (Choi et al. 2013).

3. In-situ conservation

A total of 9 conservation fences for rare and endemic species which have only one or two habitat in Korea have been installed and monitored by the KNA as part of in-situ conservation (Table 1; Son 2015). Most of all, the population sizes have been increased continuously after the installation of up-to-date conservation fences. In addition, the KNA has carried out a re-introduction program for rare plants such as epiphytic orchids in natural habitats by developing propagation techniques with accurate genetic tags.

Table 1 The current status of conservation fences for *in-situ* conservation by KNA.

No.	Taxon	Year of installation	Region
1	*Forsythia saxatilis* (Nakai) Nakai	2008	Gangwon Province
2	*Caragana fruticose* (Pall.) Besser	2008	Gangwon Province
3	*Prunus choreiana* H. T. Im	2008	Gangwon Province
4	*Ribes komarovii* Pojark.	2008	Gangwon Province
5	*Cypripedium guttatum* Sw.	2009	Gangwon Province
6	*Habenaria radiate* (Thunb.) Spreng.	2010	Gyeonggi Province
7	*Cypripedium japonicum* Thunb.	2012	Gangwon Province
8	*Veronica pusanensis* Y.Lee	2013	Busan Metropolitan City
9	*Lychnis wilfordii* (Regal) Maxim.	2014	Gangwon Province

4. International cooperation and network

The KNA also promotes trainings and international cooperation programs for the *in-* and *ex-situ* conservation activities for the East Asian biodiversity. The KNA has managed a national and public arboretums joint research council since 2010. In addition, they held the IUCN Red List Training Workshop in 2011. The KNA also attempts to encourage citizens' interests in rare and endemic plants in Korea by means of various exhibition events with a topic of rare and endemic plants in Korea.

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Mangrove Conservation Genetics

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Abstract

Mangrove forests occupy a narrow intertidal zone of tropical and subtropical regions, an area that has been drastically reduced in the past decades. Therefore, there is a need to conserve effectively the remaining mangrove ecosystems. In this mini-review, we discuss how recent genetic studies may contribute to the conservation of these forests across its distribution range at different geographic scales. We highlight the role of mangrove dispersal abilities, marine currents, mating system, hybridization and climate change shaping these species' genetic diversity and provide some insights for managers and conservation practitioners.

Introduction

Humans are changing the world at an alarming rate and at the global scale. These changes are sufficient to leave their stratigraphic, geochemical and radiogenic signatures in the geological records (Waters et al. 2016). Additionally, the biosphere also presents signs of human activities such as human-driven vertebrates extinction rates, which are much higher than conservative "background" rates (Ceballos et al. 2015). Understanding and addressing problems concerning the loss of biodiversity are the main goals of conservation biology. It focuses on the application of different fields of knowledge, from sociology to ecology and evolutionary biology, to preserve the biological diversity from the genetic to the biome level (Soulé and Soulc 1985; Wilcove 2009). The variation at the DNA level is particularly important because it is the foundation of the evolutionary processes. With that in mind, conservation geneticists use a vast arsenal of techniques to describe patterns of genetic variation or to make inferences about evolutionary processes concerning different organisms, mainly rare and endangered species. This is particularly relevant when an entire community is under threat, as is the case of mangroves.

Mangrove forests occupy the intertidal zones of tropical and sub-tropical regions (Tomlinson 1986), and its distribution has been drastically reduced in the past decades (Valiela et al. 2001; Duke et al. 2007). These tree communities are naturally composed by fewer species than other tropical and subtropical forests (Tomlinson 1986). 11 of the 70 true mangrove species (sensu Tomlinson 1986) are considered Critically Endangered (CE), Endangered, or Vulnerable according to the International Union for Conservation of Nature (IUCN) Red List categories of threat, whereas seven species are considered Near Threatened species (Polidoro et al. 2010). By describing the genetic diversity and understanding the natural or "human-made" evolutionary processes that generated this variation, it may be possible to detect early signs of population reductions and then reduce the changes of species extinction. This is only one of the many contributions of genetics to the conservation of mangrove ecosystems.

In this mini-review, our main objective is to discuss some of the recent mangrove conservation genetics related papers and highlight species differences and commonalities of evolutionary patterns and processes to provide some insights concerning mangrove conservation. Our focus on recent efforts is justified by the existence of an inspiring review (Triest 2008).

Mangroves as colonizer species

Colonization, as the establishment of a given species in a site that it has not previously occupied, is a general feature of all organisms. However, the extent

and scale that this process occurs varies among organisms and mangrove species are specially adapted for colonizing new regions (Tomlinson 1986). As a likely adaptation to an intertidal habitat between land and sea, most mangrove species share three traits: buoyant and salt-water resistant propagules (i.e. dispersal units), and embryos that develop while they are still attached to the mother tree without dormant periods (Tomlinson 1986). These features allow mangrove plants to travel long distances through water so that transoceanic dispersal has been reported for different genera both in the western (Nettel and Dodd 2007; Takayama et al. 2013; Cerón-Souza et al. 2015; Mori et al. 2015a) and eastern hemispheres (Takayama et al. 2013; Lo et al. 2014). From the management point of view, incorporating long distance dispersal qualitatively or quantitatively into population models would likely improve conservation programs' success (Trakhtenbrot et al. 2005).

Additionally, considering these 'dispersal' traits, one would also expect that mangrove propagules would majorly flow through prevailing ocean surface currents. It implies that, on the geographic scales from hundreds to thousands of kilometers, the seawater surface movement would act as both gene flow maintainer and barrier depending on the populations' geographic location. In the complex land and seascape of South-East Asia, the congruence between gene flow patterns and the predominant ocean current was reported for different genera (Chiang et al. 2001; Su et al. 2006; Liao et al. 2006; Yahya et al. 2014; Wee et al. 2014; Wee et al. 2015). Similarly, in the eastern coast of the Neotropical region, the bifurcation of the South Equatorial Current into North Brazil and Brazil currents seems to be a key driver of population subdivision (Pil et al. 2011; Mori et al. 2015b). Accordingly, land masses play an important role as barriers to the gene flow in mangrove species both between the western and eastern hemisphere and within each of these biogeographic regions (Triest 2008; Takayama et al. 2013; Lo et al. 2014; Sandoval-Castro et al. 2014; Wee et al. 2014; Cerón-Souza et al. 2015). Therefore, due to this apparent general feature among mangrove species, conservation programs that focus on this geographic scale should not ignore the population genetic connectivity driven by ocean currents and land masses. Inputting these factor in metapopulation models (Ouborg et al. 2010), for example, would be particularly interesting.

On smaller scales, conversely, mangrove dispersal is comparatively limited considering gene flow by both pollen and propagules. Although there are differences among species due to their natural history and ecological traits (Cerón-Souza et al. 2012), generally there is genetic structure on a local scale even when no obvious constrains exists (Geng et al. 2008; Islam et al. 2012; Cerón-Souza et al. 2012; Mori et al. 2015b). It may lead to a pattern of spatial genetic structure (Geng et al. 2008; Islam et al. 2012; Cerón-Souza et al. 2012). One impressive example of how limited mangrove pollen and propagule dispersal may be on a local scale is the dispersal distance of only tens of meters as estimated for a viviparous species from the eastern hemisphere (Geng et al. 2008). This limited dispersal is likely linked to the self-compatibility and mixed mating system that some mangrove species present (Landry and Rathcke 2007; Geng et al. 2008; Cerón-Souza et al. 2012; Nadia et al. 2013; Landry 2013; Mori et al. 2015b). Collectively, these results have many conservation implications; for instance, in view of the limited pollen and propagule dispersal on local scales, deforestation of an area may imply an irreversible genetic diversity loss even within a single estuary (Cerón-Souza et al. 2012).

Hibridization is a major evolutionary process in mangrove species

Gene flow occurs not only among populations but also among species ,and its consequences are quite diverse (Hoffmann and Sgrò 2011; Abbott et al. 2013). In mangroves, ongoing and/or ancient hybridization has been reported for most of true mangrove genera: *Acrostichum* (Zhang et al. 2013), *Avicennia* (Mori et al. 2015a; Mori et al. 2015b), *Bruguiera* (Sun and Lo 2011), *Ceriops* (Tsai et al. 2012), *Lumnitzera* (Guo et al. 2011), *Rhizophora* (Cerón-Souza et al. 2010; Duke 2010; Lo 2010; Takayama et al. 2013), and *Sonneratia* (Zhou et al. 2005; Qiu et al. 2008; Zhou et al. 2008). The widespread occurrence of so many hybrids in mangrove lineages poses challenges to managers and policy makers. First, it is difficult to classify individuals identified as hybrids according to criteria of origin (natural or anthropogenic) and the presence or extent of introgression (Allendorf et al. 2001). Moreover, it is often difficult to determine the direction of hybridization, which influences on the species extinction risks (Todesco et al. 2016). Despite the call for the protection of non-anthropogenic hy-

brids of some authors (Allendorf et al. 2001), hybrids should not be included in the IUCN Red List whatever their origins may be, to avoid making things even more complicated to managers and stakeholders. For mangrove conservation professionals, the case of *Bruguiera hainesii*, currently under the critically endangered (CE) IUCN category, is quite interesting. According to genetic data, it is a hybrid between two widespread species: *B. gymnorhiza* and *B. cylindrical* (Ono et al., 2016). Although removing the label of CE may reduce this species/hybrid protection, it may also allocate any available budget to the protection of its parental species and this decision is definitely not trivial to make.

Climate change

Although hybridization is a natural process that occurs among mangrove species lineages, its occurrence may increase as the climate changes and geographic distribution of related species shifts (Hoffmann and Sgrò 2011). This is only one of the consequences of global climatic alterations to mangrove forests. Despite the ability of mangrove forest to adjust to the sea level rise (Krauss et al. 2013), in many areas, the rate at which seas are rising exceeds the soil elevation gain (Lovelock et al. 2015). Moreover, the velocity of climate change is projected to be the particular high in mangrove forests (Loarie et al. 2009) and extreme changes are expected to happen earlier in mangroves than in other environments (Beaumont et al. 2011). Consequently, evidences of geographic expansion abound in the recent literature (Osland et al. 2013; Cavanaugh et al. 2014; Saintilan et al. 2014; Crase et al. 2015; Cavanaugh et al. 2015). Predicting how mangroves will respond to the current climate changes in medium and long terms is a huge challenge. Genetic studies may contribute to this matter by understanding how selective neutral and non-neutral variations are distributed in populations. This is currently feasible due to relatively recent DNA sequencing revolution, which made possible the use of the genome-wide information and even whole-genome sequencing of non-model organisms (Ellegren 2014; Andrews et al. 2016). The information regarding species gene functions may be assessed by transcriptome analyses and, for some mangrove species, this is already available (Dassanayake et al. 2009; Liang et al. 2012; Huang et al. 2012; Huang et al. 2014; Yang et al. 2015).

Perspectives

For decades, the body of knowledge concerning the mangrove species genetic variation has attracted the attention of many researchers from different groups all around the world (Triest 2008). We expect that this trend will continue and there will be much more genetic information from more mangrove taxa and, possibly, fully sequenced genomes in the near future. However, the translation of this growing field of research into management projects and conservation policies is still a great concern (Laikre 2010; Shafer et al. 2015). For species that occur in many different political units (countries, states, municipalities), such as mangrove trees, the link between policy and research seems even weaker. Despite the existence of political instruments and treaties that appeal to the mangrove conservation (Polidoro et al. 2010), the area covered with mangrove forests still decreases (Richards and Friess 2015). Moreover, the list of 15 countries that contain 74.3% of the world's mangrove areas is composed of 14 developing countries, where environmental policies are often neglected or disregarded (Giri et al. 2011). Therefore, we urge an equal focus on both "genetics" and "conservation" in mangrove conservation genetics.

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DNA Barcoding, Environmental DNA and an Ongoing Attempt of Detecting Biodiversity in Lake Kasumigaura

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Abstract

Conservation of freshwater ecosystem is in urgent need because it provides abundant ecological services to humans. Data of biodiversity obtained from a long-term monitoring are essential for planning conservation activities. However, since species identification needs expertise in taxonomy, collecting biodiversity data under a standardized monitoring protocol requires a great effort to overcome taxonomic difficulty. Recently developed methodologies using DNA barcoding and environmental DNA (eDNA) are expected to overcome the difficulty in a long-term monitoring. DNA barcode is a nucleotide sequence of a genome that has good resolution in species identification. DNA barcoding allows us to identify species even without taxonomical expertise. DNA extracted from environmental samples such as soil and water is called eDNA, that contains DNA of a variety of organisms from microbes to macroorganisms. Analysis of eDNA yields two kinds of data: (i) occurrence and abundance data of specific organisms obtained by species-specific primer and quantitative PCR, (ii) a list of a wide variety of species inhabiting an environment, obtained by species-universal primer, next generation sequencer and a reference database of DNA barcodes. By applying those methodologies to biological monitoring of freshwater, data could be kept in high quality for a long period. Moreover, records of biodiversity can be retrospectively analyzed from eDNA, because eDNA can be semi-permanently stored. Now we are installing the eDNA methodology on the long-term monitoring of Lake Kasumigaura. For detecting biodiversity of animals in the lake, a preliminary sequencing of cytochrome *c* oxidase subunit I (*COI*) of eDNA in the lake water was conducted. As a result, species of zooplankton were detected, but benthos and fish were hardly detected. This suggested that sequences of benthos and fish should be analyzed by other techniques such as the use of specific primers, which is different from the analytical methodology of zooplankton.

Introduction

Although knowledge of ecosystem dynamics has been accumulated in recent years, predicting a changing ecosystem and proposing conservation plans for it are still challenging. To increase reliability of ecosystem estimation and validation for the conservation activities, a long-term monitoring of ecosystem is needed. However, keeping collecting meaningful data, especially those of biodiversity, at multiple sites and/or at certain frequency, takes a great deal of effort; for it requires expertise in the identification of diverse organisms. In terms of convenience and objectivity, a recently developed methodology using DNA sequences offers a solution for biological monitoring, especially for water monitoring. In this paper, we first mention the issue of biodiversity monitoring in freshwater ecosystem. Second, we describe the applicability of environmental DNA (eDNA) for monitoring of freshwater biodiversity and the importance of DNA barcoding that is essential for analyses of eDNA. Finally, some preliminary results from our ongoing eDNA survey in Lake Kasumigaura presented.

Biodiversity of freshwater

Although a number of ecosystems on earth are facing an urgent necessity of conservation of biodiversity, conservation of freshwater ecosystem is behind that of terrestrial and marine ecosystems. The monitoring data of the increase and decrease in vertebrates populations from 1970 to 2010 (Living Planet Index = LPI) showed that decreased populations were 76% in freshwater species, and 39% in both terrestrial and marine species (WWF 2014). Freshwater ecosystem accounts for small areas on earth; 0.8% of the earth surface and 0.01% of water. However, it is biologically diverse; 6% (100,000 species) of described species, one third of vertebrate species, and 40% (13,400 species) of fish species inhabit freshwater (Dudgeon *et al.* 2006). Biodiversity of small organisms such as plankton and benthos in freshwater is expected to be quite high, but it is considered to be underestimated partly because of their difficulty in morphological identification.

In biodiversity monitoring, morphological identification of organisms for data classification generally needs expertise even within small taxa. This identification methodology highly relies on an observer's skill, and even for an expert, identification of smaller organisms requires considerable time and energy. Moreover, we should carefully handle the data obtained from morphological identification because it sometimes lacks objectivity due to conflicting and different interpretation of taxonomical keys. These disadvantages set limits on the quality and objectivity of monitoring data for biodiversity to be standardized for a long period or multiple sampling sites in a broad range of area at the same time. In addition, some reports pointed out that a recent decrease in the number of taxonomists might cause the difficulty in securing experts in taxonomy and training new taxonomists (Hopkins and Freckleton 2002, Joppa *et al.* 2011).

Recent analyses of eDNA from water using DNA barcodes are expected to compensate for the disadvantages of morphological identification. Using eDNA from water would decrease the difficulty of sampling and species identification and increase the objectivity of its data. In the following section, we will describe the present situation of DNA barcoding and the recent application of eDNA for detecting biodiversity.

DNA barcoding

In this decade, DNA barcoding has been proposed for the objective identification of organisms (Hebert *et al.* 2003a). DNA barcode is a sequence in a certain region of a genome and it has sufficient nucleotide differences to distinguish species. DNA barcodes generally used are *16S rRNA* for bacteria (Caporaso *et al.* 2012), ribosomal RNAs and internal transcribed spacer (ITS) for eukaryotes (Pawlowski *et al.* 2012, de Vargas *et al.* 2015), cytochrome oxidase c subunit I for animals (*COI*, Hebert *et al.* 2003b) and *rbcl* or *matK* for plants (CBOL Plant Working Group *et al.* 2009). International Nucleotide Sequence Database Collaboration (INSDC) is the biggest and the most popular database for referring DNA barcode, and Barcode of life database (BOLD) systems (Ratnasingham and Hebert 2007) and Silva for ribosomal RNA (Quast *et al.* 2013) would provide more reliable data than INSDC.

The significance of identification using DNA barcode lies in its objectivity, which relies on the information of nucleotide sequences. Whether or not one is a taxonomist, anybody who has a technique of DNA experiment is able to obtain exactly the same nucleotide sequence from the target DNA sample. Moreover, DNA sequence itself can be a powerful tool for distinguishing cryptic species that are not morphologically discriminated. Since testing machines and convenient commercial reagent kits have been developed for more general use, techniques using DNA have no longer been reserved only for some researchers. Therefore, to receive the benefit from the versatility of DNA barcoding, we should carefully handle sequence data for the next three reasons.

First, sequence data deposited in a database are not absolutely true. Users of DNA barcode are exposed to the risk of possibly invalid interpretation of taxonomy. We should carefully analyze the data by checking ecological information, confirming clusters such as barcode index number (BIN) on BOLD system (Ratnasingham and Hebert 2013), and phylogenetic analyses, especially when researchers do not have sufficient expertise in the treated group or taxa of organisms.

Second, those databases often provide only names of family, order or classes but not species, because accumulating DNA barcodes is still under way in the world. It has been noticed that promoting accumulation of DNA barcoding is an urgent issue (Jinbo *et*

al. 2011). However, research for DNA barcoding is still progressing slowly in many regions of the world and for various taxa, because great effort is needed for sampling and their morphological identification (Jinbo *et al.* 2011). Unfortunately, it can be said that accumulated DNA barcodes for Japanese species are relatively scarce despite a number of research on molecular phylogeny and biogeography. According to Union of Japanese Societies for Systematic Biology (2003), the number of described Japanese biota species was 89,088 (excluding bacteria). In BOLD systems in March 2016, DNA barcodes of 6,864 species and 4,574 BINs in Japan were shown and they are only 7.7% and 5.1% of the total number of described species. In addition, the number of DNA barcodes in Japan is also relatively small when compared to other countries or taxonomic groups where DNA barcoding project has been energetically proceeding (Fig. 1). Considering the richness in endemic species and subspecies in the insular country, lack of DNA barcoding data must limit the applicability of recent developed methodologies such as metabarcoding using environmental DNA (eDNA). Therefore, for identifying more species, it is necessary to actively promote construction of DNA barcode database.

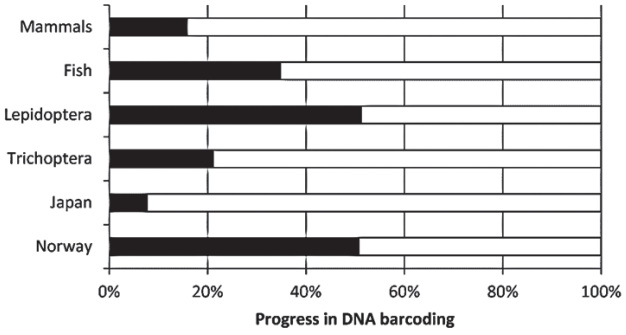

Fig. 1 Progress in DNA barcoding in the projects of taxa and countries. Black indicates frequency of species that was already DNA-barcoded. White indicates species that was described or targeted for DNA barcoding but have not barcoded yet. Data were obtained from the following sites on March 25, 2016. For mammals, iBOL mammalia barcode of life (http://www.mammaliabol.org/); for fish, Fish barcode of life (FISH-BOL, http://www.fishbol.org/); for epidoptera, Lepidoptera barcode of life (http://lepbarcoding.org/index.php); Japan, BOLD systems; Norway, Norwegian barcode of life (NorBOL, http://www.norbol.org/).

Absence of theoretical threshold for nucleotide differences distinguishing taxa often makes interpretation of taxonomy difficult. Genetic distance between species is empirically shown to be under 10 %, which in general depends on their taxa and gene regions (Hebert *et al.* 2004, Moritz and Cicero 2004, Jinbo *et al.* 2011). Therefore, if the genetic distance between sequences of two species differs by more than 10%, those species can be distinguished from each other. Conversely, when an unknown sequence of a species is perfectly matched to that of an identified species, both species can be considered as identical. However, when the difference of genetic distance is apart from 0 or 10 %, it often requires great effort to determine whether or not those species are matched. Hence, the intermediate difference of genetic distance between sequences should be carefully treated on the basis of specialized knowledge on taxonomy and molecular phylogeny.

Besides the above points, researches on biodiversity using DNA barcoding, especially metabarcoding using NGS, provide us extreme amount of information with amazingly high efficiency (de Vargas *et al.* 2015, Leray and Knowlton 2015). On the other hand, we emphasize the continuous efforts of taxonomists even though DNA barcoding research proceeds. Expertise of taxonomist is absolutely essential for accumulation of reliable information of DNA barcoding. Although most DNA barcodes used at present include taxonomical information, they do not have ecological or plastic traits such as color, shape and behavior, which are generally found by taxonomists.

Environmental DNA

Environmental DNA (eDNA) is an inclusive term for DNA extracted from environmental samples such

as water and soils (Bohmann *et al.* 2014, Barnes and Turner 2015, Thomsen and Willerslev 2015). Environmental samples are supposed to include whole body of microorganisms such as bacteria and planktons and also pieces of tissues, secretion and stools of larger organisms inhabiting the environment (Barnes and Turner 2015). The eDNA extracted from only a small amount of an environmental sample is filled with sequence information of wide range of organisms than had been expected before (Ficetola *et al.* 2008, Thomsen *et al.* 2012, Thomsen and Willerslev 2015). Recent analyses of eDNA related to biodiversity fall into two major groups. The one analytical methodology is the detection of one to several specific species by using species-specific primers and quantitative PCR (qPCR). This methodology has often been applied to detect the distribution of endemic species (Fukumoto *et al.* 2015) or invasive species (Takahara *et al.* 2013, Uchii *et al.* 2015). Since a bottle of water is enough for extracting eDNA, less damage will be caused in the field survey and less effort can be needed for the field work. Moreover, the data obtained by qPCR using specific primers for a target species can show not only its presence of species qualitatively but also its biomass quantitatively, as Yamamoto *et al.* (2016) reported that the abundance of Japanese jack mackerel could be estimated even in a bay. The other analytical methodology, called metabarcoding, sets the target at a broad range of organisms and exhaustively detect biodiversity in an environment by obtaining massive sequences using universal primers for different species and next-generation sequencer (NGS) (de Vargas *et al.* 2015, Miya *et al.* 2015). This methodology is less quantitative than qPCR, because its efficiency in PCR amplification with universal primers differs among species. However, this universality of primer annealing also enables us to detect unexpected species and the universality of metabarcoding with eDNA provides massive information of biodiversity including rare and unrecognized species. In both methodologies, integrity of the database of DNA barcodes is essential to design specific or universal primers and refer unknown sequences.

One of the advantages of this biological monitoring methodology using eDNA is the compact space for the storage of eDNA samples in micro tubes or computerized data of sequences. Moreover, DNA samples and sequence data can be stored semi-permanently in laboratories. In the traditional field studies, preservation of organismal samples often needs harmful substance and space for storage and therefore samples or data of non-target or unrecognized organisms at the treatment are generally neglected and not stored for a long time. In contrast, an eDNA sample and its sequence data from metabarcoding involve information of non-target or unrecognized organisms at the time of sampling and we are able to reanalyze them retrospectively. Simple sampling process is also the advantage of monitoring by eDNA, because sampling only a small volume of water needs no special technique. This easy sampling process enables to increase the number of monitoring sites and frequency of monitoring. Some recent reviews of eDNA illustrated the detection and analysis of eDNA and some reviews and articles also discussed unsettled problems and arguments on the reliability of eDNA (Bohmann *et al.* 2014, Rees *et al.* 2014, Barnes and Turner 2015, Rees *et al.* 2015, Roussel *et al.* 2015, Thomsen and Willerslev 2015).

Monitoring Lake Kasumigaura

Lake Kasumigaura is the second largest lake in Japan and located in the Kanto Plain where Tokyo and other large cities gather together. The lake provides us important ecological services such as water resources for drinking, agriculture and industries, and fishery, and leisure and purification of water. This close relationship of water with humans strongly affects the lake, and serious problems such as declining quality of water, blooms of blue-green algae, and invasion by alien species have been raised for many years. To understand the mechanism of ecosystem in Lake Kasumigaura, a research group of National Institute for Environmental Studies has monitored water quality and biodiversity since 1970's. Qualitative and quantitative data of bacteria, phytoplankton, zooplankton, benthos and fish are publically released on the website (http://db.cger.nies.go.jp/gem/moni-e/inter/GEMS/database/kasumi/index.html). Since it is difficult to keep standardized level of the taxonomic resolution for such a broad range of organisms for a long period, we have started to apply eDNA for the monitoring. In the rest of this paper, we will show preliminary data for animals obtained from metabarcoding of eDNA in Lake Kasumigaura and provide our perspectives on the outcome from the data analysis.

Figure 2 shows an example of metabarcoding of eDNA extracted from 250 ml water of Lake Kasumigaura on Oct. 6, 2014. The PCR reactions amplified with universal primers for *COI* gene target for animals were analyzed using NGS (IonPGM, Life Technologies). Although universal primers for animals were used, bacteria and phytoplankton were also identified. For animals, zooplanktons of Branchiopoda, Maxillopoda and Monogononta, and benthic insects were detected. Those detected zooplankton covered the main species in Kasumigaura (data not shown) and this result suggested the practical use of eDNA for monitoring biodiversity of zooplankton. On the other hand, more than 75% of contigs were not identified by Blast search. There must be a few chimeric sequences that were not removed at the assembling process, and lack of DNA barcodes in the database was considered to mainly cause the "unknown" contigs. Therefore, we are now promoting the DNA barcoding of organisms that live in Lake Kasumigaura, and the data of algae and chironomids are sequentially published on the database.

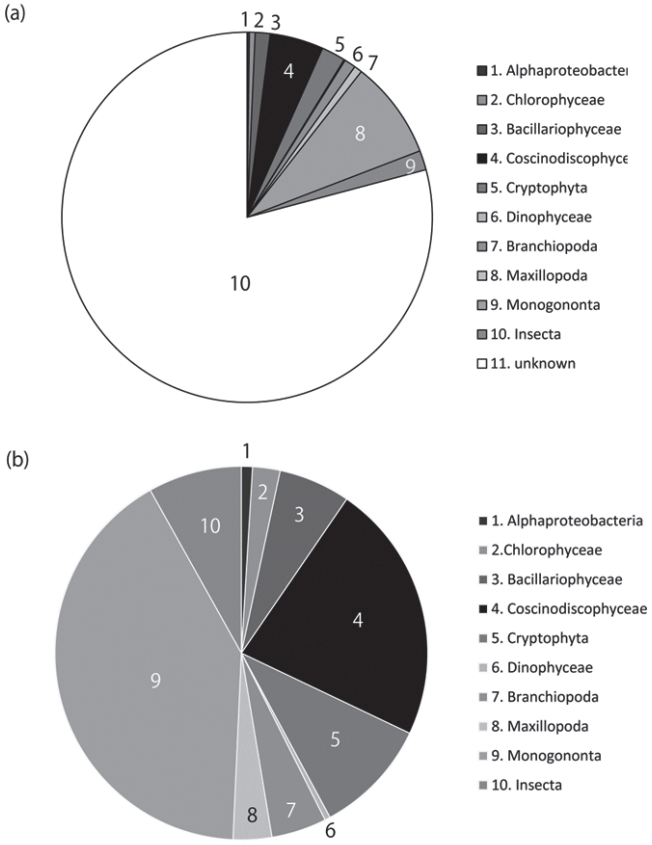

Fig. 2 Taxa detected in eDNA of water from Lake Kasumigaura. The *COI* sequences were assembled and identified by Blast search in the program package, Claident (Tanabe 2013). (a) All contigs, (b) contigs excluding unknowns.

The benthic organisms accounted for a large part of their biomass, but rarely detected from the metabarcoding of eDNA from water sample. This result might be caused by low concentration of their DNA in eDNA samples and relatively low efficiency in PCR amplification with the universal primers when compared with zooplankton. For further investigation, we will examine the water depth for sampling and design more compatible primer sets for chironomid and oligochaete species that were reported to have the largest proportion of biomass at the bottom of Lake Kasumigaura.

Fish were hardly detected from the metabarcoding of *COI* using animal universal primers (Fig. 2), which might be due to less quantity of DNA than zooplankton and inefficiency of annealing with universal primers used in this study. Although the primer set was confirmed to amplify *COI* sequences in more than

90% of fish species in Kasumigaura (data not shown), the relative efficiency would be lower than other organisms such as zooplankton. Therefore, metabarcoding of fish species from eDNA should be conducted by other systems using specific primers for fish, such as MiFISH (Miya *et al.* 2015).

Metabarcoding can be applied not only to eDNA of water body, but also to merged samples or DNA samples of individual organisms. Either the DNA solutions extracted all together from individual specimens or the mixture of DNA samples that were individually extracted from each specimen could be subject to metabarcoding of NGS (Yu *et al.* 2012). We also had preliminary data of metabarcoding for the mixture of DNA samples extracted individually from 71 species of Japanese chironomids. We mixed up the DNA samples of different concentration in equal volume into one tube and their *COI* was amplified with universal primers used above. We analyzed two replicates of PCR reactions and obtained 36 species from 47,860 reads and 29 species from 117,240 reads, respectively (Fig. 3). In total, 39 species (55%) were detected, while 32 species (45%) were not detected (Fig. 3). The correlation between the number of contigs and concentration of DNA was not significant (P=0.7983, Fig.4), and as same as the dataset excluding species without detected contigs (P=0.6931). The result might reflect the different affinity of universal primers among species rather than the concentration of each DNA sample used in the mixture. From our preliminary data, we found out that metabarcoding of DNA mixture needed improvement. Developing the primer set must be effective and exchanging the order of mixing DNA and PCR could be another way to

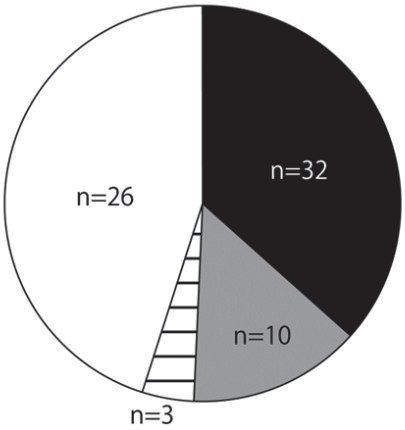

Fig. 3 Success in species identification of chironomids from the pooled DNA using NGS. DNA samples of each species were extracted individually and mixed with each volume. The result of two replicates is shown. In total of 71 species, species detected in both replicates are shown in black, those detected only in replicate 1 and 2 are shown in gray and stripes, respectively, and white shows species that were not detected from neither of replicates.

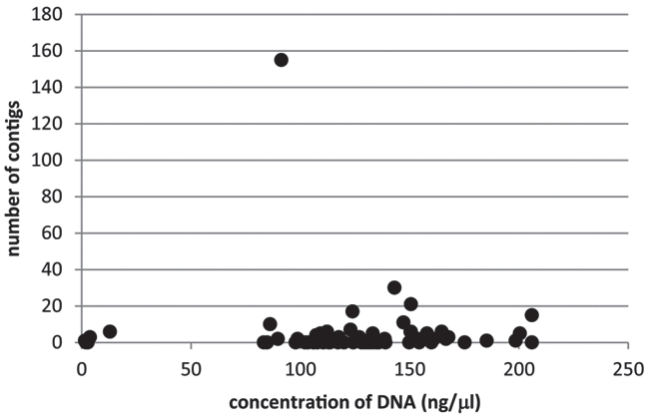

Fig. 4 Concentration of each DNA and its detected number of contigs in the NGS analysis of mixed DNA samples shown in Fig. 3.

improve the efficiency of detection.

There are several points of particular concern for the practical use of eDNA to monitor Lake Kasumigaura. First, a pore-size of filters and amount of filtering water must be critical, which were already presented in previous studies (e.g. Turner *et al.* 2014, Barnes and Turner 2015). In general, eDNA of water sample was extracted from a filter that traps eDNA and microbiota through filtration of the water sample. In most of those studies, filtering water with the pore size of 0.2 μm was recommended, but those filters were easily clogged before going through enough amount of water sample from Lake Kasumigaura. Second, the efficiency of detecting biodiversity should be considered in the application of eDNA to a long-term monitoring. Since there is no perfect primer set to detect all taxa at one time, minimal number of primer sets for target biodiversity should be investigated. For other general issues such as contamination, spatial and temporal scales should be also discussed (Thomsen and Willerslev 2015).

In recent years the efficiency and sensitivity of monitoring using eDNA have become more recognized. However, this does not necessarily mean the replacement from the traditional monitoring methodology based on morphology to the new monitoring methodologies. Applying eDNA in combination with DNA barcoding would enhance the efficiency of detecting biodiversity, which traditional monitoring has not achieved. On the other hand, the abundance and biomass of species are well estimated from the traditional sampling of organisms itself compared with those novel methodologies, at least for now. DNA barcodes can tell us the name of organisms, whereas there are important traits that are not seen by DNA barcodes. The progress in the analysis of eDNA will overcome those difficulties in the future. Until then, it is necessary to understand both advantages and disadvantages of monitoring based on tradition or eDNA and handle them properly.

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The 13th International Symposium on Integrated Field Science

Conservation and Utilization of Biodiversity

Date: March 9-11, 2016

Venue:
Lecture House no.1, Amamiya Campus, Graduate School of Agricultural Science, Tohoku University, Sendai, Japan

Hosted by
Field Science Center, Graduate School of Agricultural Science, Tohoku University, Japan

Organizer:
Yoshihisa Suyama
Associate professor
Laboratory of Forest Ecology, FSC,
Graduate School of Agricultural Science, Tohoku University

Program

Thursday, March 10

Registration

9:30–

Opening remarks

9:45–9:50

Invited Presentations

9:50–10:20 **E. A. JAMES** (Royal Botanic Gardens Victoria, Australia)
The Application of Genetics in Balancing the Conservation and Utilisation of Biodiversity in Multi-Use Environments

10:20–10:50 **Hyeok Jae CHOI** (Changwon National University, Korea)
Conservation Activities on Korean Rare and Endemic Plants —with a special reference to the Korea National Arboretum

10:50-11:00: Coffee Break

11:00–11:30 **Gustavo Maruyama MORI** (Agência Paulista de Tecnologia dos ronegócios, Brazil)

Unveiling the Genetic Variation of the Western Hemisphere Mangrove Genus *Rhizophora*

11:30–12:00 **Yuji ISAGI** (Kyoto University, Japan)
Conservation Genetics with Information from NGS in the Bonin Islands, a UNESCO World Heritage Site

12:00–13:00: Lunch

13:00–13:30 **Natsuko Ito KONDO** (National Institute for Environmental Studies, Japan)
DNA Barcoding, Environmental DNA and an Ongoing Attempt of Detecting Biodiversity in Lake Kasumigaura

Oral Presentations

13:30–13:45 **Hiroshi TOMIMATSU** (Yamagata University, Japan)
Identifying Life-History Processes behind the Abundant-Center Distribution of a Forest Herb along a Latitudinal Gradient

13:45–14:00 **Masakazu N. AOKI** (Tohoku University, Japan)
Are the Epiphytic Animal Communities in the Sargassum Forests off the Pacific Coast of Miyagi Recovering from the Alteration Caused by the 2011 Tsunami?

14:00–14:15 **Chika TADA** (Tohoku University, Japan)
Decentralized Energy Production System by Anaerobic Digestion Using Organic Waste and Exhaust Heat

14:15–14:30 **Yoshihisa SUYAMA** (Tohoku University, Japan)
Single-Pollen Genotyping Using the Next-Generation Sequencing

14:30-15:00: Coffee Break

Poster Preview
15:00–15:30
Poster Presentations
15:30–16:30
Closing Remarks
16:30–16:35
Reception
18:00–

Poster Presentations

1. C. Yonezawa et al.	Tohoku University	Aerial Measurement of Radiation Dose Distribution on Grassland Area in Kawatabi Field Science Center
2. S. Hano & Y. Kanayama	Tohoku University	Basic Study for Increasing Functional Food Ingredients Content in Tomato Using Genetic Diversity
3. H. Nariyama & Y. Kanayama	Tohoku University	Study on the Regulation of Cell Division Potentially Involved in Fruit Size Diversity in Tomato
4. H. Ohira et al.	Fukushima University University of Tsukuba	Molecular Phylogenetic Analysis of Japanese Soil-dwelling Mundochthonius Pseudoscorpions (Pseudoscorpiones: Chthoniidae)
5. S.Takizawa et al.	Tohoku University The Japanese Society for the Promotion of Science, Japan	Pretreatment of Paper Sludge with Rumen Fluid to Enhance Biogas Production
6. M. Umetsu et al.	Tohoku University	Observation of the Electrode Surface Microbes in Microbial Fuel Cells
7. R.Tajima et al.	Tohoku University	Predicting Yield, Flowering and Harvesting Dates of Highbush Blueberry Using Temperature Data: a Case Study in Field Science Center of Tohoku University
8. M.Fushimi et al.	Tohoku University Forestry and Forest Product Research Institute The Hokkaido University Museum Hokkaido University	Local Genetic Differentiation within Rebun Island in Cypripedium macranthos var. rebunense Revealed by Genome-wide SNP Analysis Using MIG-seq
9. T. Kanno & Y. Suyama	Tohoku University	Population Genetic Analysis for Identifying Hybrid Origin of a Dwarf Bamboo Species in Sasaella
10. K. Fujita et al.	Tohoku University	Conservation Genetics of Three Endangered Species of the Genus Oxera in the South of New Caledonia
11. T. Tanno et al.	Tohoku University	Plant Paleogenetics with Plant Macro-Remains from the Last Glacial Maximum

The Application of Genetics in Balancing the Conservation and Utilisation of Biodiversity in Multi-Use Environments

E. A. JAMES

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Natural systems are under increasing environmental pressures from human-mediated modification and a changing climate. Understanding the influences on plant abundance and distribution can assist in the management of these systems. Many Australian regions are experiencing lower rainfall and higher temperatures that will make some habitats less suitable in the future for species that occur there now. The use of genetic techniques can assist in promoting the evolutionary processes essential for ecosystem function and the services ecosystems provide. I will provide examples of studies where genetic techniques have enable us to understand patterns of genetic diversity by identifying maternal lineages, differentiating between individuals and clone mates and accurately measuring differences in genetic variation between individuals, populations and species. In conjunction with field-based information, better understanding of the patterns of diversity is leading to integrated management plans designed to balance the multiple pressures imposed by our use of resources with the essential requirements for biodiversity conservation.

Conservation Activities on Korean Rare and Endemic Plants - with a Special Reference to the Korea National Arboretum

Hyeok Jae CHOI[1] and Sungwon SON[2]

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The current status of conservation activities on the Korean rare and endemic species was reviewed with a special reference to the Korea National Arboretum (KNA). In the Korean peninsula, there are 4,172 plant taxa including 360 endemics and 571 rare plants categorized at the national level by the IUCN criteria: EW 4, CR 112, EN 199, LC 70, and DD 112. The KNA has established various *in-* and *ex-situ* conservation programs in practice for GSPC goals since 2010. In order to improve the conservation activities, the KNA has managed specialized research projects as well as networking programs with other local governmental arboreta and NGOs in Korea. The main purposes of the research projects are to (1) survey, update, and evaluate the conservation and genetic status of Korean populations of rare endemic plants; (2) formulate an urgent conservation strategy; and (3) monitor the endangered populations based on the latest and accurate ecological and biological information. For the *ex-situ* conservation, the KNA has secured and propagated seeds and living collections of rare and endemic plants, and as a result, the KNA conserves about 70% of Korean rare plants as living collection and propagules. A total of nine conservation fences have been installed and monitored by the KNA as part of *in-situ* conservation. In addition, the KNA has carried out a re-introduction program for rare plants such as epiphytic orchids in natural habitats by developing propagation techniques with accurate genetic tags. The KNA also promotes training and international cooperation programs for the in- and *ex-situ* conservation activities for the East Asian biodiversity.

Unveiling the Genetic Variation of the Western Hemisphere Mangrove Genus *Rhizophora*

Gustavo Maruyama MORI[1,2,3], Mariana Vargas CRUZ[2], Stephanie Karenina BAJAY[2], Koji TAKAYAMA[4], Yu MATSUKI[5], Rafael Silva OLIVEIRA[6], Yoshihisa SUYAMA[5], Anete Pereira de SOUZA[2,6], Maria Imaculada ZUCCHI[1] and Tadashi KAJITA[7]

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Global climate changes (GCC) are alterations on the climate properties whose biological effects are unquestionable. Different organisms, however, differently respond to these changes and mangrove forests are projected to be one of the most influenced environments. Understanding how and to what extent mangrove species responded to past climate alterations may provide clues to the prediction of their responses to the current GCC. Currently, our main objective is to describe the genetic structure of *Rhizophora* species from the Western hemisphere. Our results show that the two recognized species from this hemisphere, *R. mangle* and *R. racemosa*, and their putative hybrid compose a more intricate species complex. Moreover, considering only *R. mangle*, which is widely distributed across this biogeographic region, we observed that the American continent is an incomplete barrier to gene flow whereas oceans may provide pathways for long distance dispersal. We also aim to couple this information with species distribution modelling and niche analyses to unveil the past demographic history of these *Rhizophora* species across their distribution range. This project is part of a comparative and multidisciplinary study that uses genetic, transcriptomic, ecophysiological and ecological niche analyses to unveil the role of climate and its related environmental factors in the evolution and ecology of mangrove species from the western hemisphere.

Conservation Genetics with Information from NGS in the Bonin Islands, a UNESCO World Heritage Site

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[2]Faculty of Symbiotic Systems Science, Fukushima University, Japan
[3]Center for Environmental Biology and Ecosystem Studies, National Institute for Environmental Studies, Japan

The Bonin (Ogasawara) Islands are typical oceanic islands, located in the North-Western Pacific, ca. 1,000 km south of the main Japanese Archipelago. The land is dominated mainly by dry forests and sclerophyllous shrublands, and more than 440 native vascular plant species, including 70% endemics, are growing. As for land snail, 90% of 100 recorded native species are endemic. Outstanding examples of ongoing evolutionary processes evidenced by high levels of endemism and speciation by adaptive radiation can be observed in the Bonin Islands. Because of the combination of the high levels of endemism and significant adaptive radiation, the Bonin Islands were designated as a World Natural Heritage in 2011. As is often the case with oceanic island ecosystems, the Bonin Islands had been disturbed by human activities such as deforestation and introduction of invasive alien species.

In order to construct rational and effective conservation measures, we have been trying to understand/monitor the current status of the biodiversity by using genetic information from NGS. Genetic analyses for plant and bird species endemic to the Bonin Islands uncovered genetic structure, existence of unknown wild plants, unexpected feeding habitat of endangered birds, etc.

DNA Barcoding, Environmental DNA and an Ongoing Attempt of Detecting Biodiversity in Lake Kasumigaura

Natsuko Ito KONDO[1], Megumi NAKAGAWA[1], Shin-ichiro MATSUZAKI[1], Ryuhei UENO[1], Kenzi TAKAMURA[1] and Hiroshi ITO[2]

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Freshwater ecosystem is small in scale on earth but biologically diverse. Human activities induce a number of threats such as habitat loss and alterations in water quality, and a massive decrease in the population of freshwater organism is now becoming a more serious issue than terrestrial and marine lives (WWF 2012). Since freshwater provides various ecosystem services, understanding ecosystem function and mechanism that maintain ecosystem services in freshwater is essential.

In Lake Kasumigaura, National Institute for Environmental Studies (NIES) has been monthly collecting data on biodiversity as well as on water quality for over 40 years. Lake Kasumigaura in the Kanto Plain is the second largest lake in Japan and located about 60km north from Tokyo. It provides abundant ecosystem services such as water resource and fisheries, but has been threatened by declining quality of water, emergence of blue green algae and invasion of alien species. Monitoring data of Kasumigaura is used to evaluate the effects of anthropogenic disturbance on the lake ecosystem. For biological monitoring, abundance of bacterioplankton, phytoplankton, zooplankton, benthos and fish was surveyed with morphological identification. Since morphological identification requires expertise in taxonomy and takes enormous time even for experts, keeping the resolution of identification, especially at a species level, has been the problem.

Recently developed DNA barcoding and analyses of environmental DNA (eDNA) is one solution to compensate for the difficulty in the current monitoring methods. eDNA is a generic term for DNA extracted from environmental samples such as water and soils that contain DNA from whole bodies of tiny organisms and secretion or tissue fragments of larger organisms. To detect biodiversity in the lake, we evaluated the possibility to use DNA sequences from eDNA samples of the lake. For the reference of sequences from eDNA, sequence database of COI, a standard region of DNA barcoding for animals, has been built on fish, chironomids, and zooplankton of Lake Kasumigaura. We analyzed the COI fragment from eDNA amplified with animal universal primers by next generation sequencer and found that the resolution of biodiversity is high enough for zooplankton, but insufficient for larger organisms such as benthos and fish. The effect of filtration on detected sequence diversity and the importance of database were also indicated. For practical use of eDNA for a long-term monitoring in the lake, improvement of laboratory protocols will be discussed.

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Identifying Life-History Processes behind the Abundant-Center Distribution of a Forest Herb along a Latitudinal Gradient

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Understanding the processes that underlie geographic variation of population abundance has implications for determinants of geographical range limits. In addition, for assessing the potential influence of future climate change on organisms, macroclimatic variation along latitudinal gradients is relatively underutilized compared to altitudinal gradients. We compared the abundance and multiple fitness components across 23 populations of a forest herb, *Trillium camschatcense*, along a latitudinal gradient in northern Japan, from central Iwate (southern range limit) to northern Hokkaido. Flowering plant density was highest at the mid-latitude populations (~43 °N) and became progressively lower toward range limits. While the average size of flowering plants and seed production increased with latitude, the average seed mass and recruitment rate of juveniles tended to be greatest at the mid-latitude populations. A preliminary analysis showed that flowering plant density strongly positively correlated with habitat suitability predicted by an ecological niche modeling with climate variables, suggesting that demographic processes are likely to be affected, either directly or indirectly, by future climate change. Although our results suggest that reduced reproduction and recruitment play key roles in restricting the distribution of *T. camschatcense* at its range limits, the contribution of these processes to population dynamics requires further investigation.

Are the Epiphytic Animal Communities in the *Sargassum* Forests off the Pacific Coast of Miyagi Recovering from the Alteration Caused by the 2011 Tsunami?

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Sargassum forests usually harbor abundant epiphytic animals such as small crustaceans and molluscs. Most of the epiphytic animals are major food items for coastal fish and work as an important component of the marine forest ecosystem. Therefore, the abundance and diversity of these animals can be key indicators of the environmental state of *Sargassum* marine forests.

We have conducted monitoring surveys of the epiphytic animal communities in *Sargassum* beds at five localities of Shizugawa Bay in June of 2011, 2012 and 2014, and at six localities of Onagawa Bay in September and December 2012, March and July 2013, and July 2014. The survey aimed to show the temporal changes of the communities after the tsunami caused by the 2011 Pacific coast Earthquake. Samples were collected from the apical 20 cm of four different plants in a seaweed bed of dominant *Sargassum* species at each locality. All epiphytic animals retained on 1.0 mm, 0.5 mm and 0.1 mm mesh sieve were classified into eleven taxonomic groups (foraminiferans, nematodes, gastropods, bivalves, polychaetes, mites, ostracods, harpacticoids, isopods, caprellids and gammarids) and individually counted. Biomass of each taxonomic group was estimated from the size group category based on the sieve mesh size and abundance. Post-tsunami data in Shizugawa and Onagawa were compared with the pre-tsunami data of *Sargassum* epiphytic animal communities from 29 sites across Japan (data from the Ministry of the Environment, 2008).

In the post-tsunami epifauna communities on the whole, the abundance was higher and the diversity was lower than in the pre-tsunami communities. On the other hand, in the estimated biomass, no clear difference was found between pre- and post- tsunami communities. The data suggest the predominant immigration of limited number of taxonomic groups in small size (such as harpacticoid copepods) at the earlier stage of the post-tsunami period. From the temporally changes of the post-tsunami community data, the post-earthquake Shizugawa communities seem to be recovered from the alteration by the tsunami by June 2014, but not in the Onagawa communities by July 2013. Further monitoring surveys will be needed for following up the recovering process of the epiphytic animal communities, in particular, in the Onagawa *Sargassum* beds.

Decentralized Energy Production System by Anaerobic Digestion Using Organic Waste and Exhaust Heat

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Following the 2011 Tohoku earthquake, various obstacles to the activities of life were encountered when the electrical power supply was disrupted. One particular crisis arose when the function of waste treatment system stopped. We studied the construction of a decentralized energy production system by anaerobic digestion using organic waste and exhaust heat. The purpose of this project is to ensure energy supply at the time of a disaster. Biogas energy is produced via methane fermentation from organic waste using anaerobes. Electrical power is produced from generators powered using biogas. The goal of our study is to ensure energy production and resource recycling within a local area.

Large amount of fishery waste from marine product processing industry was discharged. Fishery waste was attractive material for anaerobic digestion because of its high organic matter content. However, it is a protein-rich substrate, and its degradation products, ammonia, inhibit activity of anaerobic microbes. In this study, to decrease ammonia inhibition on anaerobic microbes, the effect of oyster shell on methane gas production from anaerobic digestion of fishery waste was investigated. In addition, the energy balance for running the system was calculated. The results showed that oyster shell was enhancing methane production from anaerobic digestion of fishery waste . Various methanogen and anaerobic bacteria had attached on the oyster shell. The reasons why the gas production was enhancing by adding oyster shell was considered that oyster shell contributed as a microbial carrier in the system. Energy balance for running the system will be plus by using exhaust heat from factories and the electrical generator to warm anaerobic digestion tank.

Single-Pollen Genotyping Using the Next-Generation Sequencing

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Due to the limited amounts of DNA in a single-pollen grain, a regular use of the next-generation sequencing (NGS) has limited their adoption to pollen DNA analysis. However, recent development of PCR-based method to construct NGS libraries has opened new opportunities in NGS analysis of pollen DNA. Recently we have developed an effective method for constructing NGS libraries and genotyping of genome-wide single-nucleotide polymorphism (SNP) using NGS platform that termed "multiplexed ISSR genotyping by sequencing" (MIG-seq). Using the MIG-seq technique, thousands of genome-wide regions can be effectively amplified from a wide variety of genomes without prior genetic information. Unlike standard NGS methods based on restriction enzyme steps that require relatively large amounts of high-quality DNA, the MIG-seq procedure is based on PCR that can be applied to small amounts of DNA materials. We applied this technique to modern *Hemerocallis* pollen and successfully detected the pollen DNA data. In addition, whole-genome amplification (WGA) technique is another choice to analyze the limited amounts of pollen DNA. Once enough amounts of pollen DNA are supplied by WGA, they can be used for NGS analysis. We applied the WGA method to *Pinus* pollen grains found in a glacier, and successfully amplified pollen DNA. Combined the WGA with MIG-seq techniques, we also detected numbers of SNPs from the *Pinus* pollen grains which can be used for population genetic analysis in the past populations. These approaches would be suitable to only well-preserved pollen materials; however, we should take on challenges for the possible materials.

Aerial Measurement of Radiation Dose Distribution on Grassland Area in Kawatabi Field Science Center

Chinatsu YONEZAWA , Shin-ichiro OGURA and Masanori SAITO

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Fukushima Daiichi nuclear disaster triggered by the 2011 Tohoku earthquake released a large amount of radionuclides. Serious radioactive pollution was caused by this disaster on wide areas in the eastern part of main island of Japan. Integrated Terrestrial Field Station affiliated with Graduate School of Agricultural Science, Tohoku University, called as Kawatabi Field Science Center (FSC), located 150 km far from the nuclear power plant, is placed in the radiation hot spot area. Large grazing pastures (total 105 ha) are included in FSC and cattle were grazing there before the disaster. After the disaster, the cattle grazing has been restrained because of radioactive pollution. Most of the grazing areas are located on alpine areas with complex topography and include a variety of wild plants and small woods. Therefore, complete radiation decontamination all over the area is difficult.

In this study, we tried to estimate spatial distribution of radiation dose in a grassland area on Kawatabi FSC. An aerial measurement was applied to observe wide area at once, and obtained data was converted to a ground level by the assumption of decay rate with height as exponential curve. The air radiation dose rate was observed from a paramotor with flying ca. 500 m high to cover the target area, by a CsI scintillator for 50–300 m intervals. The radiation dose rate at the ground surface was also measured under eight paramotor measuring points and a calibration factor was computed. The radiation dose rate measured by the paramotor was 0.003–0.007μSv/h and that on the ground surface was 0.05–0.08 μSv/h. The ground level radiation was computed based on the paramotor measurements and radiation dose map was estimated by interpolations.

The generated radiation dose map showed heterogeneous spatial distribution of radioactive pollution. The computed ground level radiation distributed from 0.03 to 0.13μSv/h. The calculated value includes the indication error at the time of measurement. However, the generated map is feasible for estimating relatively high radiation dose areas. Another approach to the target area, e.g. observation using Unmanned Aerial Vehicle (UAV) is one of the effective ways to make the generated radiation dose map robuster.

Basic Study for Increasing Functional Food Ingredients Content in Tomato Using Genetic Diversity

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Introduction

Functional food ingredients have recently attracted attention because of their contribution to the maintenance and improvement of human health and to the prevention of lifestyle diseases. Tomato, *Solanum lycopersicum*, contains functional food ingredients such as lycopene and ascorbic acid, which have anti-oxidant capacity, and serotonin, which—according to a recent report—may have anti-obesity effects. To increase the content of these useful ingredients via breeding and/or cultivation methods, information on their genetic diversity and their synthesis mechanisms is essential. In this study, we investigated functional food ingredients in a tomato introgression line, IL8-3, in which a chromosomal segment from tomato wild species *S. pennellii* is introgressed into the cultivated tomato *S. lycopersicum* chromosome 8, and we also examined the synthesis mechanism of serotonin at the molecular level.

Materials and Methods

First, we measured the anti-oxidant capacity, which is measured as oxygen radical absorbance capacity (ORAC), ascorbic acid content, and serotonin content in IL8-3 and M82—the parent cultivar of IL8-3—fruit. Second, we focused on serotonin because tomato is comparatively rich in this novel and unique functional food ingredient. Serotonin content was measured during fruit development with mRNA levels of the tomato homolog genes of tryptophan decarboxylase (*SlTDC1* and *SlTDC2*) and tryptamine 5-hydroxylase (*SlT5H*), which may play roles in serotonin synthesis. Transgenic tomato plants overexpressing *SlTDC1* with *35S* promoter of cauliflower mosaic virus were developed and investigated.

Results and Discussion

Because domestication narrows the range of genetic diversity in cultivated tomatoes, wild species are useful for drastically improving fruit traits. ORAC, ascorbic acid content, and serotonin content were higher in IL8-3 fruit than in cv. M82 fruit, although their content is not always stable. Therefore, the chromosome segment form *S. pennellii* can be useful for increasing the functional food ingredients content in fruit. The serotonin content increased with fruit development and the *SlTDC1* and *SlT5H* expression levels were high in developing fruit. *SlTDC1* expression levels corresponded with the serotonin content during fruit development. These results suggested that *SlTDC1* and *SlT5H* play roles in serotonin synthesis in tomato fruit, and that *SlTDC1* is particularly important. Tomato plants overexpressing *SlTDC1* showed increased serotonin content, indicating that *SlTDC1* plays a key role in serotonin synthesis. The diversity in tomato SlTDC genes will be of interest in future studies as one of the determining factors of serotonin content.

Study on the Regulation of Cell Division Potentially Involved in Fruit Size Diversity in Tomato

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Introduction

Tomato exhibits genetic diversity in fruit size, shape and color—factors that are useful for breeding. Generally, fruit size depends on cell division and cell expansion, and fruit development consists of a cell division period and a cell expansion period. Therefore, we focused on the regulation mechanism of cell division as one of the determining factors for fruit size. In *Arabidopsis* roots, HIGH PLOIDY 2 (HPY2) induced by auxin reportedly regulates cell division. In this study, auxin and anti-auxin treatments and the expression analysis of *SlHPY2*—a tomato homolog gene of *HPY2*—were performed to reveal the effect of auxin on cell division in tomato fruit.

Materials and Methods

For auxin and anti-auxin treatment, tomato 'Ailsa Craig' fruit was dipped into 1-naphthylacetic acid (NAA) every day from day 4 post anthesis, or into anti-auxin every day from anthesis. Fruit diameter was measured at days 8 and 15 post anthesis. Paraffin sections prepared from their pericarp were dyed, and the number of cells per unit length in the mesocarp was measured. Total RNA was extracted from the fruit and *SlHPY2* expression was measured through quantitative real-time PCR.

Results and Discussion

In NAA treatment, the number and size of cells did not differ between NAA-treated fruit and the control on day 8 post anthesis, whereas the number of cells was larger and the cell size was smaller in the treated fruit than in the control on day 15 post anthesis. In the expression analysis, *SlHPY2* mRNA levels did not differ between NAA-treated fruit and the control on day 8 post anthesis, whereas the levels rose with NAA concentration on day 15 post anthesis. In the anti-auxin treatment, the number of cells was smaller and the cell size was larger in the treated fruit than in the control on both days 8 and 15 post anthesis; however, *SlHPY2* mRNA levels did not differ between the anti-auxin treated fruit and the control. These results suggest that auxin promotes cell division, and that further study is necessary to elucidate the role of SlHPY2.

Molecular Phylogenetic Analysis of Japanese Soil-dwelling *Mundochthonius* Pseudoscorpions (Pseudoscorpiones: Chthoniidae)

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The pseudoscorpion genus *Mundochthonius* is currently composed of 24 species across the Holarctic region. In Japan, three soil-dwelling *Mundochthonius* species have been described: *M. japonicus* Chamberlin, *M. kiyoshii* Sakayori, and *M. itohi* Sakayori. These taxa are distinguished from each other using typical characteristics of pseudoscorpion classification, i.e., body length, the chaetotaxy of carapace and abdominal tergites, and pedipal-pal femur morphology. However, recent taxonomic and phylogenetic studies of chthonioid pseudoscorpions have suggested that these morphological characteristics are not always reliable diagnoses. Therefore, the classification of Japanese soil-dwelling *Mundochthonius* species should be reconsidered. Here, we collected *Mundochthonius* specimens from the entire distributional range of the genus in Japan (Hokkaido to Kyushu region), including all type localities, and examined the phylogenetic relationship among them using partial sequences of mtDNA COI and nDNA 18S rRNA genes.

Molecular phylogenetic analysis showed the existence of more than three lineages of Japanese soil-dwelling *Mundochthonius* (Figure). *Mundochthonius kiyoshii* and *M. itohi* each formed their own monophyletic group with high bootstrap values. On the other hand, *M. japonicus* was comprised of five polyphyletic lineages (*M. 'japonicus'* sp. A, B, C, D, and E) indicating inconsistency between the current morphological identification and genetic lineages. Morphological analysis among the five genetic lineages suggested that the genetic lineages *M. 'japonicus'* sp. A, D, and E might be distinguished from other lineages based on some morphological characteristics such as carapacal length, spinneret and inner teeth morphology of chelicera, and length ratio between femur and tarsus of the first leg. Therefore, further morphological analysis among genetic lineages may provide new insight into the classification of *Mundochthonius* puseudoscorpion.

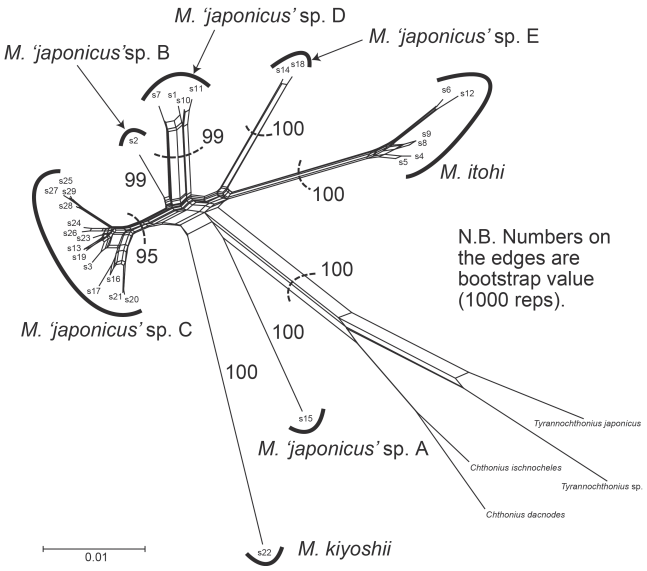

Figure Neighbor-net tree based on the haplotypes of nDNA 18S rRNA gene.

Pretreatment of Paper Sludge with Rumen Fluid to Enhance Biogas Production

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A large quantity of paper sludge is generated during papermaking. The paper sludge is difficult to be digested anaerobically due to its high levels of ash and lignin. Here, we investigated whether biogas production from paper sludge could be enhanced by pretreatment with rumen fluid. We also added up to 0.8 mg/ml sodium dodecyl sulfate (SDS) to rumen fluid at pretreatment to improve the efficiency of biogas production. Methane production from paper sludge with 6-h pretreatment was 30 times higher than that without pretreatment. This indicates that pretreatment with rumen fluid can significantly enhance biogas production from paper sludge. Upon the addition of 0.4 and 0.8 mg/ml SDS, the number of ruminal protozoa and the volume of methane production decreased. No differences in chemical oxygen demand and volatile fatty acid concentration were observed upon SDS addition. It is considered that the decrease in the number of fiber-degrading protozoa by SDS addition inhibited the degradation of paper sludge and decreased methane production. From these results, adding SDS during pretreatment using rumen fluid does not seem to have any advantages for the methane fermentation of paper sludge.

Observation of the Electrode Surface Microbes in Microbial Fuel Cells

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A microbial fuel cell (MFC) is a device that converts organic matter to electricity with the aid of microorganisms serving as biocatalysts. A typical MFC is constructed with two chambers, an anode chamber and a cathode chamber. In the anode chamber, microorganisms oxidize organic matter and transfer electrons to the anode electrode. Electrons move along a circuit to the cathode chamber, where oxygen accepts the electrons. A conventional MFC uses platinum as the cathode catalyst; however, since platinum is expensive, there is a need for an alternative catalyst at the cathode. In this study, we constructed an MFC in which both the anodic and cathodic reactions were catalyzed by microorganisms. The cathode chamber was inoculated with a hydrogenotrophic methanogen, *Methanothermobacter thermautotrophicus* strain ΔH (NBRC 100330), and sludge from an anaerobic digester was used as an inoculum in the anode chamber.

A maximum current density of 0.217 mA/cm^2 was generated by our MFC. At the end of the power generation period, the electrodes were removed and observed using a scanning electron microscope (SEM). Rod-shaped cells thought to be ΔH were observed on a gap and the surface of the cathode electrode. SEM images of the anode electrode revealed that microorganisms in various sizes and shapes were attached to the electrode. Because the metabolism of the microorganisms acts as the catalyst in the MFC, the power output may be enhanced by increasing the number of microorganisms on the electrodes.

Predicting Yield, Flowering and Harvesting Dates of Highbush Blueberry Using Temperature Data: a Case Study in Field Science Center of Tohoku University

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Blueberry is a high priced fruit crop because of its color, flavor and nutritional properties and has been increasingly cultivated in Japan. Highbush blueberry (*Vaccinium corymbosum* L.), one of blueberry species, can be grown in cool temperate regions such as hilly and mountainous areas of northern Japan. In our Field Science Center of Tohoku University, located in these areas, we have cultivated highbush blueberry since 1995 and the blueberry preserve is our leading product. However, the yield level of highbush blueberry in our center is unstable and unpredictable, probably depending on the course of spring and summer temperature. Here, we established simple growing degree-days models using temperature data and predicted the yield, the flowering and the harvesting dates of highbush blueberry.

Three highbush blueberry cultivars, Early Blue, Colins, Spartan were planted in the field of Field Science Center (lat. 38°44′30, N, 145°45′10, E, 165m elevation) in 1995 and seven cultivars (Bluejay, Patriot, Bluecrop, Blueray and three above-mentioned cultivars) were planted in 1998. We used these datasets of the yield, the flowering date, the starting and ending dates of harvesting from 1999 to 2014. We also used the datasets of the mean daily temperature (T) for model development, which were collected from AMeDAS station located next to the field.

To predict the flowering date, the starting and ending dates of harvesting using T, we established the simple growing degree-days models. In these models, if T is more than the base temperature (T_{base}), we accumulated T - T_{base} and if T is lower than T_{base}, we accumulated 0. We used 12 °C and 10 °C as the T_{base} for the flowering date and for the starting and ending dates of harvesting, respectively. In addition, for the starting date of harvesting, if T is more than 25°C, we accumulated 15°C. When the accumulated temperature reached each cultivar-unique degree, we assumed the date as the flowering date, the starting or ending dates of harvesting. To compare the predicted dates with actual ones, we used the datasets between 2005 and 2014 of highbush blueberry planted in 1998 for the exclusion of the effects of plant age. From these results, we could predict the flowering date, the starting and ending dates of harvesting using our proposed models.

Futhermore, to predict the yield using the flowering date, the starting and ending dates of harvesting, we used a multiple regression model. From this result, we could predict the yield using two durations: the duration between the flowering date and the starting date of harvesting and the duration between the starting and ending dates of harvesting. The predictions of the yield and the two durations using temperature data were not so accurate but statistically significant.

Finally, we extended the models combined with the plant growth function using plant age to apply to the immature highbush blueberry. With all datasets (1999-2014) from our field, the yield could be largely predicted except the yields were underestimated in 2000, 2011 and 2013 and the yield was overestimated in 2002. The variations of actual yield were much larger than that of the predicted yield.

In summary, we could predict the flowering date, the starting and ending dates of harvesting using temperature data of highbush blueberry and could also predict the yield with the actual data of flowering date, the starting and ending dates of harvesting. If the plant age is known, we might predict the yield in the immature plants.

Local Genetic Differentiation within Rebun Island in *Cypripedium macranthos* var. *rebunense* Revealed by Genome-wide SNP Analysis Using MIG-seq

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Cypripedium macranthos var. *rebunense* is an endangered orchid endemic to Rebun Island, Hokkaido, Japan. The natural habitats of the species are geographically separated into northern and southern regions of the island. It has been reported that the southern population showed relatively low genetic diversity by the bottleneck effect due to illegal collecting since the 1950's. Here we performed population genetic study for the species using MIG-seq analysis, PCR-based method for genome-wide SNP genotyping using the next-generation sequencing platform, to obtain more accurate population genetic data than previous one. Our study revealed that the genetic diversity within populations was not significantly different between northern and southern populations. However, northern and southern populations showed high level of genetic differentiation (F_{ST}: 0.140). Moreover, we estimated the demographic history of the populations by using approximate Bayesian computation (ABC)-based analysis. The ABC analysis suggested that (i) the divergence of northern and southern populations occurred *ca.* 7,000 years ago, (ii) population size of the southern population is much larger than previously estimated (effective population size; 1,000–2,000), (iii) the population sizes have not fluctuated since the population divergence. Thus, it is suggested that the northern and the southern populations experienced independent long-term history. Based on these results, we recommend that the both populations should be treated as independent conservation units. That is, "artificial gene flow" such as transplantation and artificial crossing between the two populations should not be conducted for conservation activities.

Population Genetic Analysis for Identifying Hybrid Origin of a Dwarf Bamboo Species in *Sasaella*

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Phylogenetic systematics of bamboo species is rather complicated possibly due to the existence of not only interspecific but also intergeneric hybrids. Therefore, studies of the genetic origin of such hybrids are required to clarify their systematic relationships. It has been pointed out that, as an example of putative hybrids, the species in the genus *Sasaella* are intergeneric hybrids between the genera *Pleioblastus* and *Sasa*, based on their morphological characteristics. Here, we conducted population genetic analysis for identifying the parental species of a putative hybrid species of *Sasaella*.

DNA samples were collected from five bamboo species; *Sasaella masamuneana* f. *hashimotoi* as a putative hybrid, *P. chino* and *Sasa palmata* as its putative parents, *Sasa spiculosa* and *Sasamorpha borealis* as sympatric species in Kawatabi Field Science Center, Tohoku University. These samples were analyzed using simple sequence repeat (SSR) markers and single nucleotide polymorphisms obtained by next-generation sequencer (NGS-SNP). (i) Genetic diversity, (ii) structure, (iii) parentage assignment analysis, and (iv) approximate Bayesian computation analysis for demographic history of the populations were conducted using the population genetic data. In addition, (v) chloroplast DNA nucleotide sequences were investigated for the putative hybrid and parent species.

(i) Based on seven SSRs and 36 NGS-SNPs, the highest value of observed heterozygosity was detected in the putative hybrid. (ii) Both of the genetic structure analysis estimated by seven SSRs and 94 NGS-SNPs showed that the putative hybrid has intermediate genetic composition between the putative parents. (iii) Parentage assignment analysis detected offspring-parents groups in the populations of putative hybrid and parents. (iv) Demographic history of the populations supported the putative hybrid originated from hybridization between populations of two putative parents of the hybrid population. (v) Chloroplast DNA haplotypes of the putative hybrid were shared by the putative parents.

These results strongly suggested that the putative hybrid of *Sasaella* is intergeneric hybrid origin between *Pleioblastus* and *Sasa*. Our study approach can be applied to investigating a wide range of hybrid species, and will be able to contribute to systematic studies of many species.

Conservation Genetics of Three Endangered Species of the Genus *Oxera* in the South of New Caledonia

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New Caledonia is a global biodiversity hotspot consisting of numerous endemic species. However, their habitat range has declined due to human activities such as nickel mining and bush fire. *Oxera baladica*, *O. pancheri* and *O. rugosa* are endemic plant species in New Caledonia and in danger of extinction. In this study, their genetic diversity, genetic structure, and demographic history of the populations of these three species were investigated based on single-nucleotide polymorphism (SNP) analysis with next-generation sequencing (NGS).

O. baladica is sparsely distributed in the north and south of the island, and south populations were focused in this study. Leaf samples were collected from 78 individuals of all the recognized populations in the south of the island. *O. pancheri* and *O. rugosa* are only distributed in the south of the island, and the samples were collected from all the recognized individuals (126 individuals from seven populations and 38 individuals from three populations, respectively). DNAs were isolated from the samples and used for sequencing and searching genome-wide SNPs.

In total, 71, 133 and 68 loci were found as SNP markers in *O. baladica*, *O. pancheri* and *O. rugosa*, respectively. Their population genetic data showed lower values of observed heterozygosities than expected in most populations of the three species, suggesting higher a level of inbreeding in each local population. Analysis of spatial genetic structure clearly showed genetic differentiation among local populations of each species. Estimated demographic history of the populations in *O. rugosa* indicated that the local populations were separated from a larger population several hundred years ago due to the effect of human impacts. Consequently, it is proposed that the each population of the three species should be conserved as independent unit. In particular, populations of *O. rugosa* have the highest priority in conservation activities against recent human impacts.

Plant Paleogenetics with Plant Macro-Remains from the Last Glacial Maximum

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Ancient DNA (aDNA) from plant remains should provide valuable information for paleoecologists as well as population geneticists; however, the rarity of well-preserved plant macro-remains has often prevented widespread adoption for plant aDNA studies. Here we report remarkably well-preserved sediments originated from the Last Glacial Maximum (LGM), and an attempt to retrieve aDNA from macro-remains in the sediments.

The sediment core was collected from Sugiyaike in the Hira Mountains, Siga Prefecture, Japan. Plant macro-remains were isolated from eight parts of the sediment core (*ca.* 1 g each) dated *ca.* 27,200 cal yr. BP. The sediment samples included a large number of small pieces of needles with some kept almost in the complete shapes. *Picea* and *Abies* needles were abundant and identifiable based on their morphology.

DNA extraction was conducted for 53 samples of each large piece or mixtures of small pieces of needles. The extracted DNA solutions were used for templates of PCR targeting a short region of cpDNA, and the PCR products were sequenced.

Three out of the 53 samples showed positive PCR amplification; however, their sequences correspond with only *Pinus*. We speculate that the inconsistency of sequences could originate from unexpected contamination; therefore, more appropriate procedures should be done for the future experiments.

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