Workshop of Young Scientists on Biodiversity and Conservation Biology in Southwest China: Advances in Conservation Genetics

中国西南地区生物多样性和保护生物学青年研讨班

保护遗传学研究进展

10 – 13 July 2015, Kunming

2015 年 7 月 10-13 日，昆明

ABOUT THE WORKSHOP

In recent decades, molecular techniques have become an integral component of conservation biology. The advent of Next Generation Sequencing (NGS) techniques opens up unique opportunities for molecular techniques to be the main tool for species identification, movement tracking, population demography monitoring and for understanding the basic ecology of threatened species. Advances in the application of NGS have thus taken center stage in conservation genetics.

This workshop focuses on the application of molecular techniques on conservation issues. The first day features a series of research projects from Southwest China and Japan on species of conservation concern. Following the seminar series, there will be a three-day training course on a newly developed NGS technique for genotyping, termed MIG-Seq. The training course will be conducted by Prof Yoshihisa Suyama and Dr Yu Matsuki from Tohoku University, Japan.

Sponsors

Kunming Branch, Chinese Academy of Sciences

中国科学院昆明分院

Kunming College of Life Science, University of Chinese Academy of Sciences

中国科学院大学昆明生命科学学院

Organizers

Xishuangbanna Tropical Botanical Garden, Chinese Academy of Sciences

中国科学院西双版纳热带植物园

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SYMPOSIUM ON ADVANCES IN CONSERVATION GENETICS

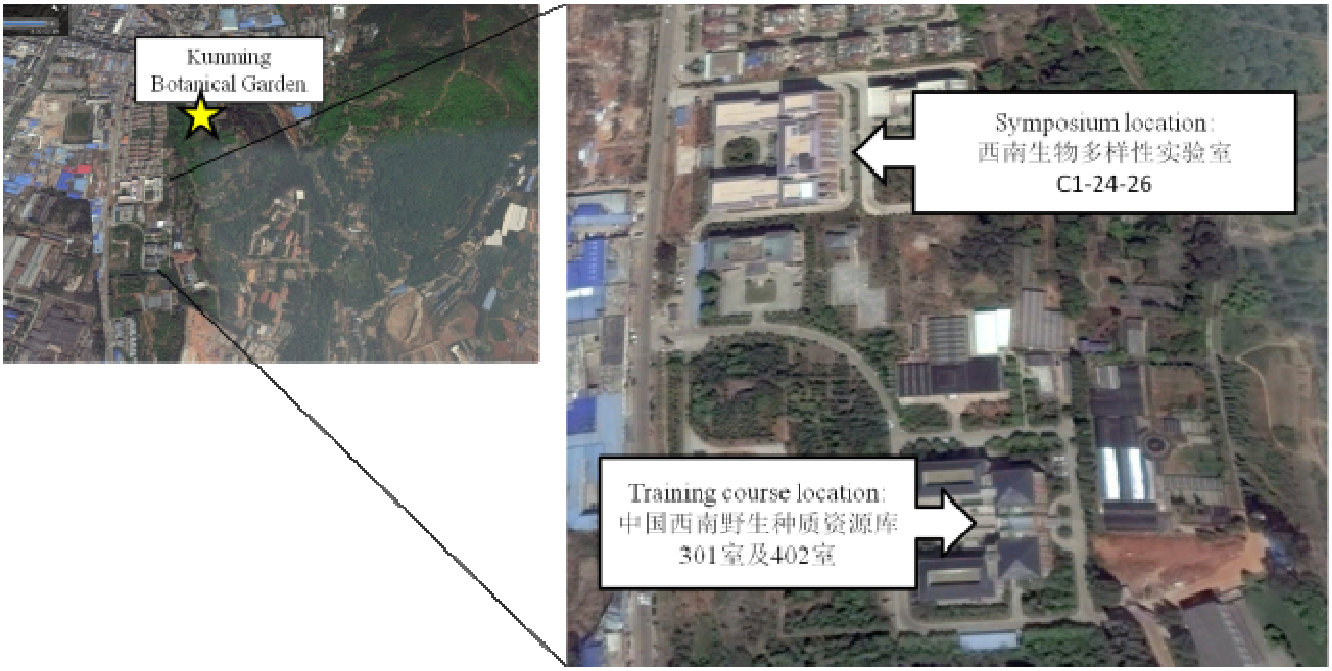
Date: 10th July

Venue: Laboratory of Biodiversity in Southwest China, conference room C1-24-26

昆明市盘龙区茨坝镇青松路 21 号

西南生物多样性实验室

实验楼 1 楼， C1-24-26 报告厅



Talks / presentations

Talks will be for 15 mins with 5 mins Q&A. Talks in English are strongly encouraged, but Chinese talks with English PPT are also welcome.

SYMPOSIUM ON ADVANCES IN CONSERVATION GENETICS

Time (AM)	Topics
09:00-09:30	**Registration**
09:30	**Welcome address** **Yongping Yang**（杨永平） Deputy Director of KIB
09:40	**Welcome address** **Jianghua Chen**（陈江华） Deputy director of XTBG
09:50	**MIG-seq: an effective PCR-based method for genome-wide SNP genotyping using NGS platform** **Yoshihisa Suyama**（陶山佳久） Kawatabi Field Science Center, Tohoku University
10:10	**Single pollen genotyping for investigation of plant-insect relationship —Pollination efficiencies of pollinator insects visiting *Magnolia* flowers** **Yu Matsuki**（松木悠） Kawatabi Field Science Center, Tohoku University
10:30-11:00	**Group photo and coffee break**
11:00	**RAD-sequencing as a tool for unravelling the population dynamics of ecologically diverse plant lineages** **Shota Sakaguchi**（阪口翔太） Laboratory of Plant Evolution and Biodiversity, The University of Tokyo
11:20	**Rescuing PSESP (Plant Species with Extremely Small Populations) in China: A case of the Yangbi maple *Acer yangbiense*** **Weibang Sun**（孙卫邦） Plant Conservation and Acclimation Research Group, KIB
11:40	**Protocol of Constructing ddRAD Library for NGS** **Zhenhua Guo**（郭振华） Comparative and Functional Genomics Research Group, KIB
12:00-14:00	**Lunch break**

SYMPOSIUM ON ADVANCES IN CONSERVATION GENETICS

Time (PM)	Topics
14:00	**The conservation biology of *Loropetalum subcordatum* (Hamamelidaceae): An endemic plant of China** **Jiuxiang Huang**（黄久香） College of Landscape Architecture and Forestry, South China Agricultural University
14:20	**Species delimitation of recently derived *Pedicularis* in the Himalaya-Hengduan Mountains using genotyping-by-sequencing data** **Wen-Bin Yu**（郁文彬） Center for Integrative Conservation , XTBG
14:40	**Genetic optimization of trees in living collections: Improving ex-situ conservation of threatened species** **Alison K.S. Wee**（黄锦嫦） Center for Integrative Conservation , XTBG
15:00-15:20	**Tea break**
15:20	**Land use change is a reality but conservation is a priority** **Kingsly Chuo Beng** Community Ecology and Conservation Research Group, XTBG
15:40	**Transcriptome analysis of *Pterospermum kingtungense* implications for endangering mechanisms** **Yandong Ren**（任彦栋） Evolutionary Genomics and Gene Origin Research Group, KIZ
16:00	***De Novo* sequencing and assembly analysis of transcriptome in sodom apple (*Calotropis gigantea*), a fiber producing wild shrub** **Nkatha Gacheri Muriira** Plant Molecular Genetics and Genetic Engineering Research Group, KIB
16:20	**Symposium closing remarks and briefing for training course**

MIG-seq: an effective PCR-based method for genome-wide SNP genotyping using NGS platform

Yoshihisa Suyama and Yu Matsuki

Kawatabi Field Science Center, Graduate School of Agricultural Science, Tohoku University

Restriction-enzyme-based next-generation sequencing (NGS) methods have revolutionized marker-assisted genetic studies; however, the use of restriction enzymes has limited its widespread adoption, especially in field samples with low-quality DNA and/or small quantities of DNA. Here, we developed a PCR-based procedure to construct reduced representation libraries without restriction-enzyme digestion steps, representing *de novo* single nucleotide polymorphism discovery, and its genotyping using next-generation sequencing. Using multiplexed inter-simple sequence repeat (ISSR) primers, thousands of genome-wide regions were effectively amplified from a wide variety of genomes without prior genetic information. We demonstrate reproducibility of genotyping by checking its applicability for clone (individual) identification and its applicability in a wide variety of species by checking standard population genetic analysis. This approach, termed “multiplexed ISSR genotyping by sequencing” (MIG-seq),is applicable to a wide range of marker-assisted genetic studies, especially for medium-scale studies based on less than 1000 markers in ecological, phylogeographic, and conservation genetics,including quick surveys of genetic differentiation among individuals (clones and breeding varieties), populations, closely related species, and hybrids.

Single pollen genotyping for investigation of plant-insect relationship —Pollination efficiencies of pollinator insects visiting *Magnolia* flowers

Yu Matsuki

Graduate School of Agricultural Science, Tohoku University

In the pollination processes of majority of flowering plants, insects play an important role for pollen transfer. Because flower-visiting insects have wide characteristics in morphological and behavioral features, different insects will differently affect on pattern of pollen movement, eventually reproduction success of pollinated plants. Pollination process is a limited opportunity of gene flow for plants, it is important to clarify the patterns of pollen movement and plant-pollinator relationship for understanding of reproduction system, and also conservation of plant species.

In this study, a novel method to analyze DNA from single pollen was developed. Using this method, the genetic composition of pollen grains that were transported by pollinator insects were analyzed. Pollen donors were assigned and finally the pollination efficiencies of each flower-visiting insects were evaluated.

The genotypes of pollen grains that adhered to the insects that visited to flowers of *Magnolia obovata* were directly determined. Most of the pollen adhered to bumblebees (Apidae, *Bombus* spp.) and small beetles (Lagriidae, *Arthromacra* sp.) was self-pollen (pollen that was transported to a different region on one tree). On the other hand, considerable part of the pollen grains that adhered to flower beetles (Scarabaeidae, subfamily Cetoniinae, *Protaetia* sp.) were transported from other reproductive trees. And we also describe the composition of pollen donors and the pollen movement patterns by the parentage analysis between pollen and reproductive trees. Our results indicate that bumblebees and small beetles scarcely move inter-plants, and might cause self pollination of *M. obovata*. Because the early stages of *M. obovata* undergo substantial inbreeding depression, visitation of those insects may negatively affect the reproduction of *M. obovata*. In contrast, flower beetles transported large amount of genetically diverse outcross pollen grains. Our evaluation is corresponding to the traditional idea that the flowers of Magnoliaceae have features of beetle pollination syndrome. Single pollen genotyping method will make up for the shortcomings of traditional approaches, and provide useful information for the problems that can be hardly explained by traditional approaches, such as the impact of human activities and climate fluctuation.

RAD-sequencing as a tool for unravelling the population dynamics of ecologically diverse plant lineages

Shota Sakaguchi

Laboratory of Plant Evolution and Biodiversity, Department of General Systems Studies, Graduate School of Arts and Sciences, The University of Tokyo

Recent advances in high-throughput sequencing techniques opened a new avenue for researchers to use enormous molecular data in population genetics/phylogenetics of plant species. Among them, ddRAD (double digest Restriction site Associated DNA) sequencing is a cost and time efficient method to prepare reduced representative library, with which we can obtain genotypes of hundreds of samples genotyped at several to tens of thousands of SNPs. The increased number of molecular markers enables us to unravel weak/fine genetic structure that cannot be detected by traditional markers, to establish high-density genetic maps and even to capture adaptive genomic regions under natural selection. In this talk, I will introduce my recent studies on ecologically diversified plant lineages revealed by ddRAD sequencing. 1) While cypress pine (*Callitris columellaris*) is a drought tolerant conifer species complex inhabiting semi- and arid regions of Australia. To construct a baseline genetic map for ecological genomics of this species, I established a single-tree linkage map consisted of over 4,000 SNPs markers. The marker segregation and distribution pattern suggested spatially variable selective pressure andrecombination rate along linkage group. Although the map was built based on polymorphism from a single tree, ca. 30 % SNPs were recovered in independent RAD-sequencing of population samples of the species, suggesting its potential to investigate detailed evolutionary history of the species complex. 2) Asian goldenrod (Solidago asiatica) shows ecological and morphological differentiation between typical forest-edge habitat and serpentine open habitatin Hokkaido Island, Japan. The Japanese goldenrod populations were previously analysed with cpDNA and EST-SSR markers, but no significant population structure was detected, which indicates recent ecological diversification occurred in this group. Recently, RAD-sequencing derived SNPs were analysed in soil ecotypic populations in Hokkaido, and successfully detected an incipient ecological speciation process, induced primarily by differentiation of flowering time between serpentine and non-serpentine habitats.

Rescuing PSESP (Plant Species with Extremely Small Populations) in China: A Case of the Yangbi maple *Acer yangbiense*

Weibang Sun, Jing Yang, Linlin Zhao, Jia Ge

Kunming Botanical Garden, Kunming Institute of Botany, Chinese Academy of Sciences

The new concept of PSESP (Plant Species with Extremely Small Populations) developed for rescuing most of the globally threatened plant species was firstly promulgated in Yunnan of China in 2005, and it's now widely accepted. Currently, several national and regional-level conservation strategies and actions (120 species targeted by State Forestry Administration and National Development & Reform Commission, and 62 species listed by Yunnan provincial government) have being implemented in China. PSESP is characterized by small remaining populations (even far lower than Minimum Viable Population, MVP), restricted habitat, serious human disturbance, and extremely high risk of extinction. And thus the "Rescuing Protections" together with the "Comprehensive Studies" are particularly important for effectively conserving PSESP. The Yangbi maple (*Acer yangbiense*) was newly described species in 2003, and only one site with the five mature individuals has been located in Yangbi county in Dali Prefecture in west Yunnan Province. As a case, this presentation will detailed introduce the current actions have being done for rescuing the Yangbi maple. And these are: 1) creating *in-situ* protection site; 2) studying reproduction biology and propagation for *ex-situ* conservation; 3) exploring the genetic diversity of remaining individuals and propagated saplings; 4) samplings for both *ex-situ* population-establishment at KBG (Kunming Botanical Garden) and the reintroduction (including the reinforcement) into the semi-natural habitats; and 5) public education.

Protocol of Constructing ddRAD Library for NGS

Guo Zhenhua , Yang Guoqian

Comparative and functional genomics, Kunming Institute of Botany, Chinese Academy of Sciences

Restriction-site associated DNA sequencing (RAD-Seq), which interrogates a fraction of the genome across many individuals, is an ideal method for plant genotyping. By using restriction enzyme digestion and sequencing the regions adjacent to restriction sites, researchers can examine the same subset of genomic regions for hundreds of individuals and identify thousands genetic markers along the genome. Until now RAD-seq has become a powerful and useful approach in genetic marker discovery, linkage mapping, phylogenetics and population genetics. Traditional RAD-Seq uses one restriction enzyme and random shearing to generate fragments from genomic DNA. However, these are high DNA loss steps and offer little control over the fragments to be sequenced. Thus, the double digest RAD-Seq (ddRAD-Seq), a variation of RAD-Seq for genotyping was implemented by Peterson *et al.* in 2012. The ddRAD protocol uses two enzymes to digest genomic DNA in a four-step protocol. Genomic DNA is simultaneously digested with two restriction enzymes (usually a low frequency cutter combined with a high frequency cutter). Barcoded P1 adapters (with an overhang matching the first restriction site) and P2 adapters (with an overhang matching the second enzyme restriction site) are ligated onto digested fragments in a single sticky-end ligation. Samples are then pooled and size selected. Finally, PCR is used to enrich the library and also to introduce a second barcode in the form of an Illumina index, increasing multiplexing potential.

The temperate woody bamboos (Arundinarieae) are of great ecological and economic importance as they provide food and raw materials for humans and numerous animals. They are highly diversed in morphology but lack a substantial amount of genetic variation. The complex evolutionary history results in the intractable taxonomy of this lineage, and the relationships within the tribe have not been well resolved. The objectives of our research were to develop orthologous loci with single nucleotide polymorphism (SNP) markers using ddRAD-Seq and to reconstruct the evolutionary relationships of temperate woody bamboos. As a pilot study of the whole project, we firstly optimized the ddRAD library construction protocol with two woody bamboo species: *Phyllostachys viridiglaucescens* (Carr.) A. et C. Riv. and *Pseudosasa japonica* (Sieb. et Zucc.) Makino. We selected AvaII+MspI as our enzymes combinations and 400-600bp as our target size when cutting the gel. A total of 13,719,139 and 1,956,307 reads were obtained separately from two species and the quality of the raw data was verified by FastQC. Through analysis with the Stacks SNP-calling pipeline, 2155 loci(13513 SNPs) showed polymorphism between *P. viridiglaucescens* and *P. japonica*. This study will lay foundation for the large scale molecular phylogeny analysis of temperate woody bamboos in the near future.

The conservation biology of *Loropetalum subcordatum* (Hamamelidaceae): An endemic plant of China

Jiuxiang Huang

College of Landscape Architecture and Forestry, South China Agricultural University

Loropetalum subcordatum is a member of genus *Loropetalum* (Hamamelidaceae), which is endemic to China with four distributed regions throughout Guizhou, Guangxi, Guangdong province and Hong Kong. The high genetic diversification of *Loropetalum subcordatum* should be result from inbreeding depression and the spread restriction of the seed. We study the seed Biology, tissue culture and observed the growth situation in field. We intend to study the ecological foundation by applying the NGS technology in future research.

Species delimitation of recently derived *Pedicularis* in the Himalaya-Hengduan Mountains using genotyping-by-sequencing data

Wen-Bin Yu

Center for Integrative Conservation, Xishuangbanna Tropical Botanical Garden, , Chinese Academy of Sciences

Species is one of the basic units of biological classification, and it is the fundamental unit for investigating biogeography, conservation, ecologyand evolution. Systematistsagree that species is real in nature, while species is very difficult to identify and describe using a general rule. Traditionally, species is delimited on the basis of one or more qualitativeor quantitative morphological characters that show nooverlap with other species. Currently, molecular data are frequently used to delimit species,where species status is determined on the basis of an exclusivitycriterium that reciprocal monophyly.Molecular methods have uncovered some cryptic species in practice. However, molecular approaches is difficult to delimit recently derived species, because some neutral DNA loci often have not had sufficient sequence variations/substitutions to achieve the monophyly.*Pedicularis* series *Reges* is recently derived species group, including two species and five infraspecific taxa. Molecular phylogenies strongly supported this series as monophyletic, while multiple samples from one taxon were not resolved as monophyletic. In this study, we use genotyping-by-sequencing (GBS)data to delimit 22 samplesrepresenting thefivemorphology-based taxa in series *Reges*. Our results showed that phylogenies resolved four recognized taxa, and an unknown taxon close to *P. rex*. In addtion, gene flow between taxa was low or not significant. Our study demonstrate that GBS data are well resolved species relationship of recently derived group in *Pedicularis*.

Genetic optimization of trees in living collections: Improving ex-situ conservation of threatened species

Alison K.S. Wee

Center for Integrative Conservation, Xishuangbanna Tropical Botanical Garden, , Chinese Academy of Sciences

Xishuangbanna is a plant diversity hotspot, supporting 10% of China's angiosperm flora. Agricultural expansion in recent decades had resulted in habitat loss and fragmentation, driving extinction in many plant species. To ensure the long-term survival of threatened plant species, the Center for Integrative Conservation in the Xishuangbanna Tropical Botanical Garden (XTBG) has initiated a "Zero Extinction Project". Central to this project is the ex situ conservation of threatened species, many of which will be included in the living collection within XTBG. Efficient management of the living collection requires that the total genetic diversity of a species is represented by the least number of individuals within the collection. Hence there is a need for a genetic screening method that is (1) rapid, (2) applicable to many angiosperm species, and (3) is sufficiently cost-effective that large numbers of seedlings can be genotyped.

A pilot study was initiated to test the genetic optimization protocol. *Aglaia teysmanniana* (Meliaceae) is an ideal focal species for the pilot study. The seedlings are easy to identify in the wild and can be found in clusters near to the mother tree. Collecting seedlings from the wild allowed us to circumvent the phonological and germination challenges of sample collection. *Aglaia teysmanniana* is listed as Near Threatened on the IUCN Red List and Vulnerable on the Zero Extinction Project assessment in Xishuangbanna.

We collected 418 seedlings from seven populations in four locations in Xishuangbanna (Figure 5 and 6). The GPS coordinate of the seedling patches were recorded, leaf samples of potential mother trees in the vicinity were collected, and the seedlings were transplanted to the XTBG nursery (Figure 7). Only 204 seedlings (49%) survived the transplantation process; root damage may be the cause of most of the mortality. The surviving seedlings will be genotyped with both RAD-seq and MIG-seq to evaluate the cost, time and labour requirements of both methods.

In summary, this study represents a large technological step forward in the ex situ conservation of tropical plant species and is essential to XTBG's position as a pioneer in plant conservation.

Land use change is a reality but conservation is a priority

Kingsly Chuo Beng

Center for Integrative Conservation, Xishuangbanna Tropical Botanical Garden, , Chinese Academy of Sciences

Continuous habitat loss and degradation is predicted to displace species from their natural habitats to human-dominated habitats, where they will be required to acclimate or adapt to new ecological conditions or face extinction. Xishuangbanna is situated in one of the World's 25 biodiversity hotspots but its status as a biodiversity hotspot is under threat from various human-induced pressures. From 1976 to 2003, total forest area reduced by 28%, whilst rubber alone expanded by 90%. Little is known about the effects of anthropogenic land-use change on the arthropods of the biodiverse tropical forests of Xishuangbanna. To evaluate the impact of agricultural expansion on arthropods, we metabarcoded all arthropods captured within habitats on 35 matched pairs of natural and human-modified habitats. We determined species richness, turnover, and community composition in all habitat types and related these to environmental correlates, such as habitat structure, leaf litterproperties and topography. Arthropod species richness decreased significantly along the land-use modification gradient. Arthropod species composition was significantly different between matched pairs of natural and human-modified habitats. Species composition patterns across the land use gradient were best explained by disturbance, topography and leaf litter chemistry and species richness by disturbance and leaf litter phosphorus. These results suggest that land use change have irreversible effects on arthropod biodiversity and natural habitats are irreplaceable for the conservation of this important group of organisms.

Transcriptome analysis of *Pterospermum kingtungense* implications for endangering mechanisms

Yandong Ren

Evolutionary Genomics and Gene Origin Research Group, Kunming Institute of Zoology, Chinese Academy of Sciences

Pterospermum kingtungense C.Y.Wu ex Hsue, which is endemic to China. This species only exists in Jingdong County, Yunnan Province, Southwest China, and is distributed within an extremely limited range. In this study, using Illumina sequencing technology, we generated 44.75million sequencing reads for seedling. These reads were assembled into 50,333 unique sequences and 44,795 sequences were annotated with an E-value above 10-5. We identified several genes that have evolved in response to positive selection between Cacao and Pterospermum kingtungense. These genes clues for its endangering mechanisms.

De Novo sequencing and assembly analysis of transcriptome in sodom apple (*Calotropis gigantea*) , A fiber producing wild shrub.

Nkatha Gacheri Muriira

Plant Molecular Genetics and Genetic Engineering Research Group, Kunming Institute of Botany, Chinese Academy of Sciences

Sodom apple (*Calotropis gigantea*), a member of family Asclepiadaceae, is a large and evergreen shrub native to continental Asia and northern Africa. As an important medicinal shrub and a fiber resource plant, there is an urgent need for investigating the molecular basis of growth and development and developing molecular markers to facilitate breeding and improvement of varieties in Sodom apples.

In this study, using the Illumina high throughput sequencing technique we obtained about 45 million 125 paired sequencing reads, and *de novo* assembled and generated 50,742 unigenes, with an average length of 859 bp and N50 of 1,733 bp. Based on similarity analyses, 21,851 (43%) unigenes were functionally annotated. In particular, many transcripts that encode for putative proteins involved in fiber and secondary metabolite biosynthesis were identified and analyzed. Various transcription factors involved in regulating plant response to abiotic stress were also identified. In addition, based on the unigene sequences assembled, 11,623 microsatellite loci were detected, which provide very useful resources for developing microsatellite molecular markers.

This study is the first report on transcriptome information in Sodom apple and provides rich gene transcript resources for conducting further studies on understanding molecular basis of fiber and secondary metabolite biosynthesis, serving the genetic improvement and resource utilization in Sodom apple.

TRAINING COURSE ON MIG-SEQ

Date: 11th – 13th July

Venue:

中国西南野生生物种质资源库

301 室 （3 楼）及 402 室（4 楼）

Date	Time	Agenda	Venue
11th July	10:00	Lecture on library preparation [Part 1]	301 室
	10:30	1st PCR	
	11:00	Visit KIB Germplasm Bank	
	12:00	Lunch break	
	14:00	2nd PCR	
	14:30	Break	
	15:00	Gel electrophoresis – prepare and run	
	15:30	Interactive session	
	16:30	Gel electrophoresis - results	
12th July	09:30	Lecture on library preparation [Part 2]	301 室
	10:00	PCR purification	
	10:45	Size selection	
	11:30	qPCR	
	12:00	Lunch	
	14:00	MiSeq - preparation	
	14:30	MiSeq - run	
	15:00	Visit Kunming Botanical Garden	
13th July	09:00	Data analysis and discussion	402 室
	11:30	Final comments and workshop closing	

Mode of instruction

The molecular work will be carried out on a bench in the wet lab. It will be video-recorded and transmitted to a screen for the class. The video will be accompanied by on-site explanation by Prof. Suyama and Dr. Matsuki.

The data analysis session will be carried out in a seminar room with a common computer, and will be projected on a screen for the class.

The software for data analysis are as below (works on Mac and Linux):

- Fastx_tool kit (http://hannonlab.cshl.edu/fastx_toolkit/)
- TagDust (http://sourceforge.net/projects/tagdust/)
- Stacks (http://creskolab.uoregon.edu/stacks/)

If you would like to try out the analysis on your own, kindly bring a Mac or Linux machine, and install the software beforehand.