Protocols for short-term training program

Microorganisms in the natural environment

Members in this program:
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Vietnam National University of Agriculture

Schedule

2023 Short-Term Training Program for "Microorganisms in the natural environment"				
	Day	Time	Schedule	Teaching staff (&TA)
13-Mar	Mon	14:00	Arriving Hanoi (VN311)	Okane, Ishiga, Sakata, and Nada
		16:00	Arriving Hotel	
14-Mar	Tue	9:00-9:30	Orientation (VNUA students' meeting time)	VNUA & UT faculties
		9:30-10:00	Lecture (Outline)	Okane, Ishiga
		10:00-12:00	Sample collection (leaves and water)	Okane, Ishiga, Sakata, and Nada
		12:00-13:00	Lunch break	
		13:00-15:30	Sample processing and microscopic observation	Okane, Ishiga, Sakata, and Nada
		15:30-15:45	Break	
		15:45-17:00	Lecture (Rust fungi and epiphytes)	Okane
15-Mar	Wed	9:00-9:30	Today's menu & Lecture	Ishiga, Okane
		9:30-12:00	Sample collection (leaves) and microscopic observation	Okane, Ishiga, Sakata, and Nada
		12:00-13:00	Lunch break	
		13:00-15:00	Expt. (DNA purification) and microscopic observation	Okane, Ishiga, Sakata, and Nada
		15:00-15:15	Break	
		15:15-16:00	Microscopic observation	Okane, Ishiga, Sakata, and Nada
		16:00-17:00	Lecture (Resarch topics)	Ishiga
16-Mar	Thu	9:00-9:30	Today's menu & Lecture	Okane, Ishiga, Sakata, and Nada
		9:30-12:00	Expt. (PCR) and microscopic observation	Okane, Ishiga, Sakata, and Nada
		12:00-13:00	Lunch break	
		13:00-1500	Expt. (Electrophoresis) and microscopic observation	Okane, Ishiga, Sakata, and Nada
		15:00-15:15	Break	
		15:15-16:00	Lecture (Resarch topics)	Sakata, Nada
		16:00-17:00	Expt. (Data analysis) and microscopic observation	Okane, Ishiga, Sakata, and Nada
17-Mar	Fri	9:00-12:00	Expt. (Data analysis) and microscopic observation	Okane, Ishiga, Sakata, and Nada
		12:00-13:00	Lunch break	
		13:00-15:00	Prepare for presentation	Okane, Ishiga, Sakata, and Nada
		14:00		Okane, Ishiga, Sakata, and Nada
		17:10		Okane, Ishiga, Sakata, and Nada
18-Mar	Sat	0:20	Leaving Hanoi (VN310)	Okane, Ishiga, Sakata, and Nada
		7:35	Arriving Narita	Okane, Ishiga, Sakata, and Nada

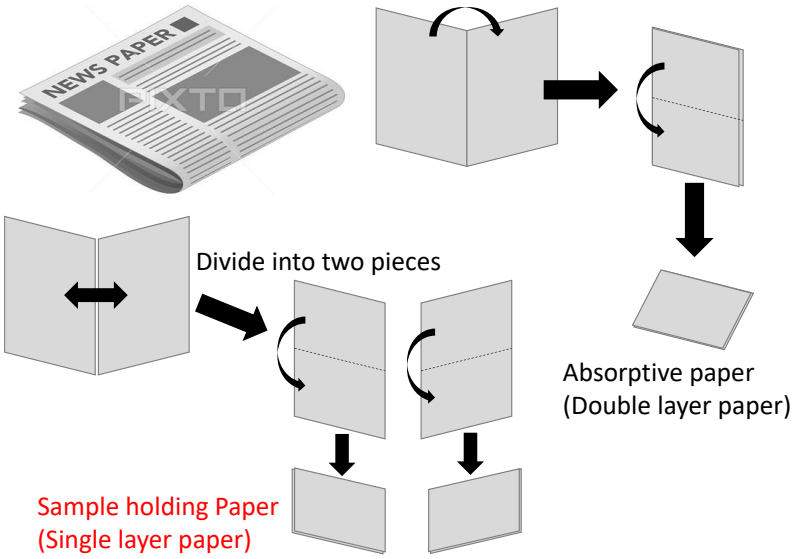
Aim and contents of practical training

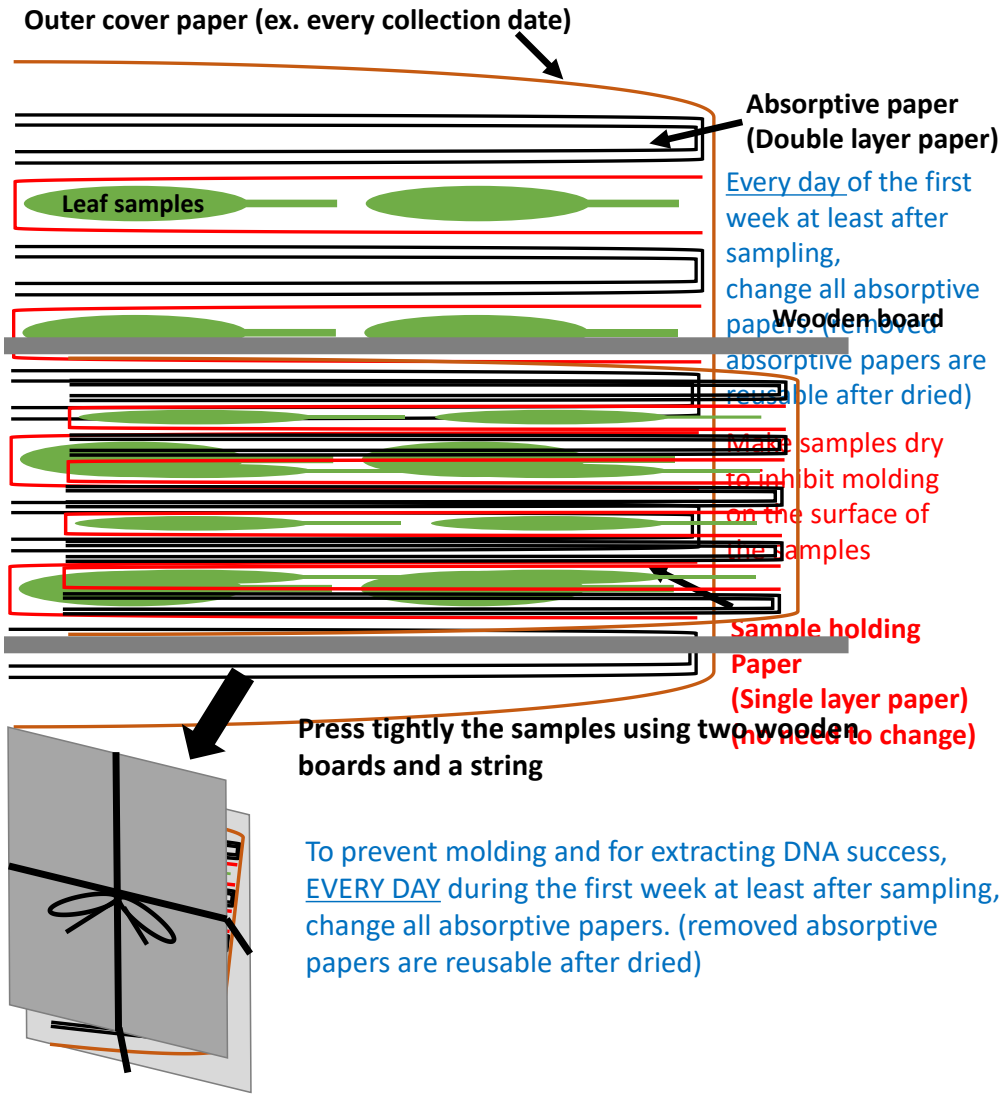
The main aim of this training course is to learn a knowledge of biodiversity of microorganisms in the natural environment and of Polymerase Chain Reaction (PCR), which is widely used in the field of molecular biology. The training program includes hands-on practical experiences for microscopy and culturing of microorganisms (fungi and Bacteria) and molecular biology, such as environmental DNA (eDNA) purification, PCR, and gel electrophoresis. This course also contains Powerpoint presentation training.

For microscopic observation and culturing of microorganisms, the training program including “Rust fungi (biotrophic obligate plant parasites)” and “Epiphytic microbes (fungi and bacteria)”. To investigate fungal and bacterial populations in natural environmental samples including plant leaves and water, eDNA will be evaluated by PCR with specific primer sets. Upon eDNA extraction, eDNA purification kit will be used. After confirming the quality and quantity of eDNA based on the gel electrophoresis, PCR will be performed to amplify the target regions with gene specific primer sets. Finally, the PCR products will be investigated by the gel electrophoresis.

Experimental procedures

1. Dried Specimen Preparation Procedure





2. Isolation of epiphytic microorganisms using washing method with aerosol OT (a surface-active agent)
    1. Wash leaves with 0.005% aerosol OT (a surface-active agent) several time and rinse with SDW for isolation of epiphytc microorganisms (and DNA extraction).
    2. Stump the surface of leaf processed on a plate of agar medium and incubate the plates.
    3. Observe microorganisms appearing on the medium plate by microscopy.

3. Sampling for eDNA extraction
    1. Soil: up to 200 mg input
    2. Water
    3. Feces: up to 200 mg input
    4. Plant: up to 20 mg
    5. Insect: up to 20 mg

6. Environmental samples you want to investigate

2. eDNA extraction from soil, water, feces, plants, and insects

DNA barcoding targeting 16S and Internal Transcribed Spacer (ITS) ribosomal RNA (rRNA) sequencing are common sequencing methods used to identify and compare bacterial or fungal populations within environmental samples.

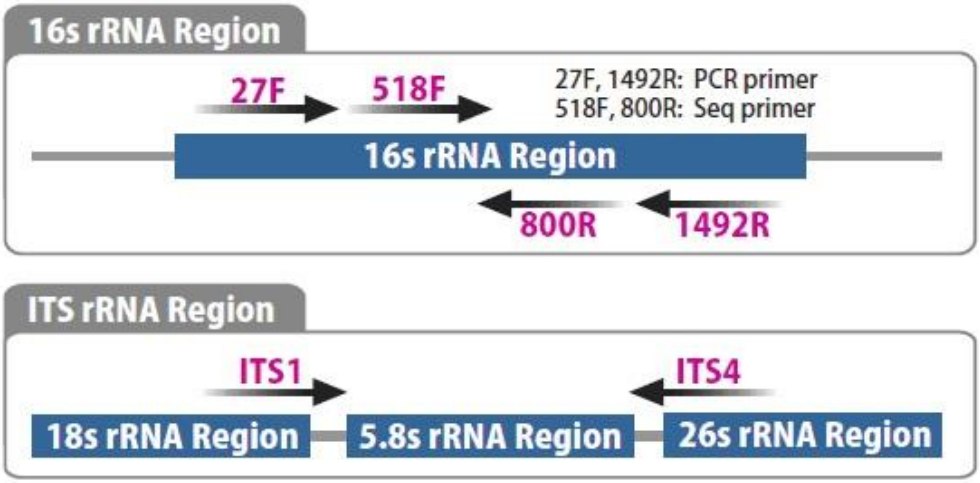


Fig. 1. Bacterial 16S and fungal ITS rRNA regions.

2.1 Materials and equipment for eDNA extraction

eDNA Mimiprep Kit (ZYMO RESEARCH), 1.5 mL tubes, 95% EtOH, pipetmans, latex gloves, vortex mixer, centrifuge.

2.2 Procedures for eDNA extraction

1. Add sample to a ZR BashingBead™ Lysis Tubes (0.1 & 0.5 mm). Add 750 µl ZymoBIOMICS™ Lysis Solution to the tube and cap tightly.
2. Secure in a bead beater fitted with a 2 ml tube holder assembly and process at maximum speed for ≥ 5 minutes.
3. Centrifuge the ZR BashingBead™ Lysis Tubes (0.1 & 0.5 mm) in a microcentrifuge at ≥ 10,000 x g for 1 minute.
4. Transfer up to 400 µl supernatant to the Zymo-Spin™ III-F Filter in a Collection Tube and centrifuge at 8,000 x g for 1 minute. Discard the Zymo-Spin™ III-F Filter.
5. Binding preparation:
   Feces and All Non-Soil Samples: Add 1,200 µl of ZymoBIOMICS™ DNA Binding Buffer to the filtrate in the Collection Tube from Step 4. Mix well.
   Soil Samples: Add 800 µl of ZymoBIOMICS™ DNA Binding Buffer and 400 µl of 95% ethanol to the filtrate in the Collection Tube from Step 4. Mix well.

6. Transfer 800 µl of the mixture from Step 5 to a Zymo-Spin™ IICR Column in a Collection Tube and centrifuge at 10,000 x g for 1 minute.
7. Discard the flow through from the Collection Tube and repeat Step 6.
8. Add 400 µl ZymoBIOMICS™ DNA Wash Buffer 1 to the Zymo-Spin™ IICR Column in a new Collection Tube and centrifuge at 10,000 x g for 1 minute. Discard the flow-through.
9. Add 700 µl ZymoBIOMICS™ DNA Wash Buffer 2 to the Zymo-Spin™ IICR Column in a Collection Tube and centrifuge at 10,000 x g for 1 minute. Discard the flow-through.
10. Add 200 µl ZymoBIOMICS™ DNA Wash Buffer 2 to the Zymo-Spin™ IICR Column in a Collection Tube and centrifuge at 10,000 x g for 1 minute.
11. Transfer the Zymo-Spin™ IICR Column to a clean 1.5 ml microcentrifuge tube and add 100 µl (50 µl minimum) ZymoBIOMICS™ DNase/RNase Free Water directly to the column matrix and incubate for 1 minute. Centrifuge at 10,000 x g for 1 minute to elute the DNA5, 6.
12. Place a Zymo-Spin™ III-HRC Filter in a new Collection Tube and add 600 µl ZymoBIOMICS™ HRC Prep Solution. Centrifuge at 8,000 x g for 3 minutes.
13. Transfer the eluted DNA (Step 11) to a prepared Zymo-Spin™ III-HRC Filter in a clean 1.5 ml microcentrifuge tube and centrifuge at exactly 16,000 x g for 3 minutes.

https://www.youtube.com/watch?v=qqi4JU8mmJo

3. Evaluating concentration and purity of eDNA

The concentration of eDNA should be determined by measuring the absorbance at 260 nm (A260) in a spectrophotometer. To ensure significance, readings should be greater than 0.15. An absorbance of 1 unit at 260 nm corresponds to 50 µg of eDNA per mL. The ratio of the readings at 260 nm and 280 nm (A260/A280) provides an estimate of the purity of RNA with respect to contaminants that absorb in the UV, such as protein and phenol. Pure eDNA has an A260/A280 ratio of 1.8–2.0.

Volume of eDNA sample = 100 µL
Dilution = 5 µL of eDNA sample + 495 µL water (1/100 dilution)
Measured absorbance of diluted sample in a 1 mL cuvette: A260 = 0.3
Concentration of RNA sample = 50 x A260 x dilution factor
= 50 x 0.3 x 100
= 1200 µg/mL

3.1 Materials and equipment for evaluating RNA

Spectrophotometer, tubes, water, pipetmans, latex gloves

4. Agarose gel electrophoresis of eDNA

The quality of eDNA preparation may be assessed by electrophoresis on an agarose gel. This will also give some information about eDNA yield.

4.1 Materials and equipment for electrophoresis

Agarose, 1 x TAE buffer, loading buffer, SYBR Green, molecular weight marker, pipetmans, latex gloves, gel electrophoresis system, gel image analyzer,

4.2 Procedures for electrophoresis

1. Make 1% agarose gel –dissolve 0.5 g of agarose into 50 mL of 1 x TAE by heating and put 2 μL of SYBR Green, and then pour into a mold with a comb when the solution cools down.
2. Add 2 μL of loading buffer into the aliquot (10 μL) of the sample.
3. Set the agarose gel on an electrophoresis apparatus (tank with 1 x TAE), load 12 μL of each samples and 5μL of molecular weight marker into each wells on the gel.
4. Run the electrophoresis for about 30 to 45 minutes at 100 V.
5. Observe the bands on the gel under blue light (Fig. 4).

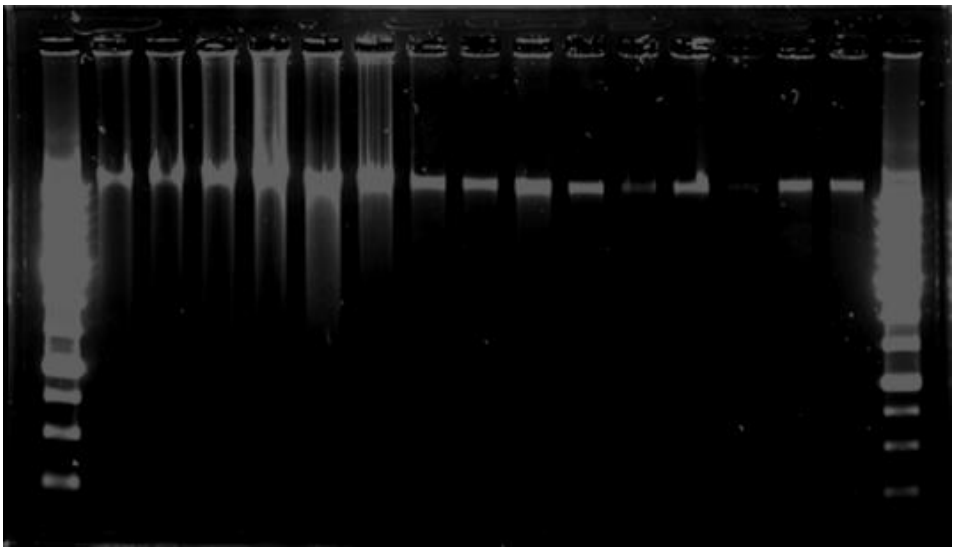


Fig. 2. eDNA from soil samples

5. PCR

5.1 Materials and equipment for PCR

EmeraldAmp® PCR Master Mix (TaKaRa), PCR forward/reverse primers*, pipetmans, latex gloves, thermal cycler, fungal and bacterial DNAs (positive control)

5.2 Procedures for RT-PCR reaction

Reagent	per reaction	final concentration
EmeraldAmp® PCR Master Mix (2x)	10 μL	1x
PCR forward primer (10uM)	1 μL	0.5 μM
PCR reverse primer (10uM)	1 μL	0.5 μM
Nuclease free water	6 μL	
eDNA (1/50 diluted) or positive control	2 μL	
Total	20 μL	

* Primer sets for RT-PCR

Gene	Description	Forward primer	Reverse primer
ITS rRNA	DNA barcoding for fungi	ITS1-F_KYO1: CTHGGTCATTTAGAGGAASTAA	ITS4: TCCTCCGCTTATTGATATGC
16S rRNA	DNA barcoding for bacteria	10F: GTTTGATCCTGGCTCA	800R: TACCAGGGTATCTAATCC

5.3 PCR program

3step PCR standard protocol

Stage 1: Initial denaturation

Cycle: none

95℃ 60 sec.

Stage 2: PCR reaction

Cycle: 35

95℃ 30 sec.

55℃ 30 sec.

72℃ 60 sec.

Stage 3: Sample storage

Cycle: ∞

10℃

6. Agarose gel electrophoresis of PCR product

The quantity of DNA can be assessed by electrophoresis on an agarose gel.

6.1 Materials and equipment for electrophoresis

Agarose, 1 x TAE buffer, loading buffer, SYBR Green, molecular weight marker, pipetmans, latex gloves, gel electrophoresis system, gel image analyzer,

6.2 Procedures for electrophoresis

1. Make 1% agarose gel –dissolve 0.5 g of agarose into 50 mL of 1 x TAE by heating and put 2 μL of SYBR Green, and then pour into a mold with a comb when the solution cools down.
2. Set the agarose gel on an electrophoresis apparatus (tank with 1 x TAE), load 10 μL of each samples and molecular weight marker into each wells on the gel.
3. Run the electrophoresis for about 30 to 45 minutes at 100 V.
4. Observe the bands on the gel under UV.