Research Activity Report
Supported by "Leading Graduate Program in Primatology and Wildlife Science"

(Please be sure to submit this report after the trip that supported by PWS.)

	2017.05.24
Affiliation/Position	Bogor Agricultural University/D1
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1. Country/location of visit

Japan/ Primate Research Institute, Kyoto University

2. Research project

Genome Science Course, Gastrointestinal parasites of mammals in Yakushima (Parasites of Wild Deer and Rodents in Yakushima: Identity, Abundance and Distribution)

3. Date (departing from/returning to Japan)

2017.05.22 – 2017. 05. 26 (5 days)

4. Main host researcher and affiliation

Dr. Munehiro Okamoto, Dr. Takashi Hayakawa (Primate Research Institute)

5. Progress and results of your research/activity (You can attach extra pages if needed)

Please insert one or more pictures (to be publicly released). Below each picture, please provide a brief description.

During Genome course, I was belong to Parasite Group. The purpose of the course are to do genetic identification of parasite collected in Yakushima Field Course. Unfortunately, we were not examine endoparasites that we found in mammals due to the small number of discovered individuals. We only concentrated to identify exoparasites (ticks) we collected from deer, rodents and ground vegetation.

The course started on 22nd May at 1 pm with the guidance of laboratory work given by Dr. Takashi Hayakawa. At that time, we met Mr. Akito Touge (PWS student at Primate Research Institute) that helped us as tutor. After guidance, we moved to laboratory at center building of PRI to start DNA extraction and purification. Before DNA extraction, we divided our sample into three group based on the habitat: deer, rodents and ground vegetation. Each group containing adult and nymph samples except for rodents because we only found nymph stage. We had to separate head and body of each ticks we found (head to morphological identification, body to DNA source) using blade under stereomicroscope. We preserved head part in 70% ethanol. The body part from each sample then crushed using Zirconia beads (@ sample using 4 beads) at Bead Smash 12 (BS-12) for 150 sec using 3200 rpm speed. We did that process twice then centrifuge for 3 minutes at 15,000 rpm. We treated the crushed sample using DNA isolation kit for tissue (QIAamp) following the protocol from the kit. On 1st day we finished our work after adding proteinase K to each sample to incubate overnight.
On 23rd, we continued DNA purification. We also performed DNA extraction using method from Ushijima et al. (2003). We used 6 samples that crushed after being frozen by liquid nitrogen and boiled for 2 minutes with 100µL of NaOH solution. After extraction and purification, we performed amplification of the 16S region of each DNA samples using PCR. We used ExTaq Hot Start Polymerase enzyme from Takara. We used 3 pairs of primer to specifically amplified the DNA (16S+1/16S-1 for 28 tick samples, LepF1/LepR1 for 2 non-tick samples, and L14724/H15915 (mammalian) for all 30 samples). Amplification was performed using the following conditions: first denaturation at 98°C for 10 min; 35 cycles of denaturation at 98°C for 10 sec, annealing at 52°C for 30 sec, and extension at 72°C for

1 min, followed by a final extension at 72°C for 10 min. Annealing temperature was set for specific primer sequences. During PCR, we pour electrophoresis gel (1% Agarose + GelGreen Mix (3µL in 100mL TAE buffer) into gel tray and let it cool and harden. We finished the work before PCR completed, and Dr Takashi Hayakawa helped us to store the PCR products in the fridge for overnight.

On 23rd May, we performed visualization of PCR products by electrophoresis. We mixed 5µL of PCR product with 1µL of loading dye and put it into each well of gel electrophoresis. We also added marker into empty wells to know the size of PCR products. We performed electrophoresis in 100V for 20 minutes. After that we put the gel on FAS-LED BOX (Nippon Genetics) and taking photo by blue LED with 520nm filter. We could only amplify 15 samples (14 samples suspected as Haemaphysalis from deer and vegetation and 1 sample suspected as Ixodes from rodents). We could not amplified samples extracted using Ushijima methods, it was suggested that DNA purification is important to get good DNA template for amplification. At the same day, we performed PCR again using DNA using half-length primers (16+1/16-2 and 16+2/16-1) for unamplified samples and 2nd PCR using full-length primer (16+1/16-1) for unamplified 1st PCR products. The electrophoresis result for our PCR showed we got five amplified PCR products (one from second half-length primer (16+1/16-2), two from first-half length primer (16+2/16-1), and two from full length primer (16+1/16-1). Those amplified 3 Haemaphysalis and 2 Ixodes. After that, we performed ExoSAP purification for PCR products to remove primers and excess nucleotides using condition: 2µL ExoSAP for 20µL PCR products incubated at 37^0C for 30 min and 80^0C for 1 min. The product of ExoSAP then processed to sequencing. Using the PCR primers (full-length primer, first half-length and second half-length primers), the purified PCR products were directly sequenced in both strand orientations with a BigDye Terminator Sequencing Kit with 30 cycles of amplification. We purified the sequence products using magnetic beads (Agencourt CleanSEQ) and performed capillary electrophoresis using 3130 Genetic Analyzer (Applied Biosystems). Dr Takashi Hayakawa explained us how to operate the 3130 Genetic Analyzer and how it works.

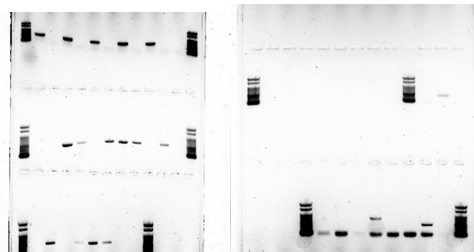
On 24th May, we were ready to analyze our sequence data, but unfortunately (again) we had to come back to laboratory work because we could not get any sequence data. We started again to purified successful PCR products using Isopropanol purification methods, the classic purification method but very powerful, instead of ExoSAP purification. Then we did sequencing again using same reaction mixtures as we did the day before. After sequencing, we purified our sequence product using EtOH purification instead of magnetic beads purification.

On 25th May, we finally success performing sequencing and get good sequence results. We started analyze our data using Geospiza Finch TV for viewing and editing trace data of Sanger sequences. Using this software we removes the noise sequence and primer sequences. After removing noise, we continued our analysis using MEGA 7 software (http://www.megasofteare.net/) for constructing alignments and phylogenetic trees. As team, we worked efficiently and we were divided to analyses and compiled the sequences, each of us worked with sequence data from 5 samples. Finally, we gather our sequences and start to align the sequences using my computer. We aligned our samples with previously reported sequences of ticks (accession number of NCBI databases available at Black and Piesman. 1994. *Proc. Natl. Acad. Sci. USA*) available at NCBI nucleotide database (https://www.ncbi.nlm.nih.gov/nucleotide/). After aligment, we constructed phylogenetic tree using Neighbor-Joining method with 1,000 bootstrap replication. The evolutionary distances were computed using the Jukes-Cantor method. We constructed three phylogenetic tree, full-length three, first-half tree and second-half tree based on primers we used to amplify the 16S region. Surprisingly, we got two cluster of suspected Haemaphysalis (that supported by morphological identification) suggested two species of new

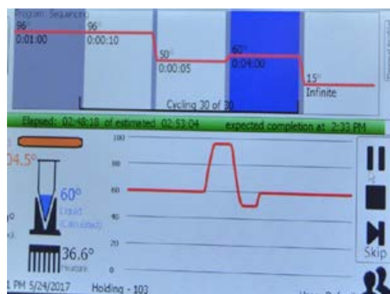
record Haemophysalis species. Those cluster do not grouped with other Haemaphysalis that previously reported. We were so happy! After constructing tree, we continued to making poster and still continue to make poster until the next day.

Besides the activities I mention above, we enjoyed Inuyama. This small town is pretty nice and Dr Takashi Hayakawa also brought us to Japan Monkey Center at 25th May, there I was so happy to see many species of primates and also animals like dog, turtle and gecko in Kids Zoo. I love delicious foods prepared by institute cafetaria. It was memorable to stay 5 days in Inuyama and see many familiar faces and meet my friends. Overall, I greatly enjoyed the field course and had an impressive experience with all my new friends and lecturers. During this field course, we learnt new skill and scientific work, did great teamwork, and help each other.

Separation of head and body of each ticks sample



Visualization of PCR products



PCR condition

Sequencing preparation

6. Others

I would like to express my gratitude to all organizing committee, staffs and researchers for their support. Especially thank Dr. Munehiro Okamoto, Dr Takashi Hayakawa, Mr Akito Touge, Mrs Umemura and my Parasite team for great help in the research and daily life. Thank all lecturers and students for their help and support during the course. I also want to express my gratitude to PWS and CET-Bio for organization and financial support.