REPORT OF THE

Two Months Attachment Program on Diagnostics of Leafminers of Agricultural Importance

at
Department of Biological Sciences, Faculty of Science,
Nara Women's University, Nara, Japan

August 01 – September 30, 2016

BY
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Thailand

Organized by:

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2016

Attachment Program: Diagnostics of Leafminers of Agricultural Importance

(JAIF Funded Project on Taxonomic Capacity Building to Support Market Access for Agricultural Trade in the ASEAN Region)

Venue: Nara Women University, Japan

Duration: 1st August to 30th September 2016

Participant Name &Position: Yuvarin Boontop (Entomologist)

Institutional Address and Country: Department of Agriculture, Phahonyothin Road, Chattuchak, Ladyao, Bangkok, Thailand

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1. Background Information

The ASEAN Plant Health Cooperation Network (APHCN)-ASEANET Project "Taxonomic capacity building to support market access for agricultural trade in the ASEAN region", funded by the Japan ASEAN Integration Fund (JAIF) successfully held its second capacity building activity, a "Training Workshop on Leafminers of Agricultural Importance", from 29th February to 11th March 2016 at Museum Zoology, LIPI, Cibinong, Indonesia. Under this ASEAN-endorsed Project, three participants from the training workshop were selected for a two-month attachment program at the laboratories of the lead resource person, Professor Hiroaki Sato at the Nara Women University, Nara, Japan, for further training. The attachment focused primarily on leafminers belonging to the order Lepidoptera.

I, **Yuvarin Boontop,** was nominated by the Project Steering Committee, based on the advice of the resource persons of the training workshop, as one of the three successful candidates for this attachment program. The starting date and details relating to the attachment program were finalized by Dr. Sato, the overall supervisor for the attachment program. I was accompanied for the entire program by another participant in the attachment program, Ms Ariene Castillo of the Agri-Food and Veterinary Authority of Singapore.

All expenditures (international travel economy class and DSA) associated with participation in the attachment program was borne by the Project and paid through ASEANET. ASEANET also facilitated processing of travel and other, associated arrangements.

2. Objectives of the Attachment

2.1 The participants in the attachment were to acquire taxonomic skills in identifying the species, genera and families of major pest leafminers, on the basis of external characters and DNA barcoding.

2.2 Participants were also to gain understanding of how seriously leafminers damage horticulture crops and familiarity with appropriate control methods.

3. Program of Attachment (Time Table)

Month	Date	Day	Activities	Notes
Aug.	1	Mon	Arrival at Kansai Airport, Japan	
			Travel to Nara Women's University (NWU)	
	2	Tue	Collect leafminers in hills of Nara City	
	3	Wed	Make preparations of leafminers in NWU	
	4	Thu		
	5	Fri		
	6	Sat		Sato, out of office due to business trip
	7	Sun		
	8	Mon	Day off	
	9	Tue	Day off	
	10	Wed	Collect leafminers in hills of Nara City	
	11	Thu	Make preparations of leafminers in NWU	National Holiday
	12	Fri		
	13	Sat		
	14	Sun		
	15	Mon	Visit to Dr Oshima's lab. of Kyoto Prefecture University to learn DNA sequencing techniques	Under supervision by Dr Ohshima
	16	Tue		
	17	Wed		
	18	Thu	Make preparations of leafminers in NWU	
	19	Fri	Visit to Dr. Oshima's lab. of Kyoto Prefecture University	
	20	Sat		
	21	Sun		
	22	Mon	Collect leafminers in hills of Nara City	
	23	Tue	Make preparations of leafminers in NWU	Dr Sato, out of office due to a special lecture and practice
	24	Wed	Collect leafminers in Mt Takama hill	
	25	Thu	Make preparations of leafminers in NWU	
	26	Fri		

Month	Date	Day	Activities	Notes
	27	Sat		
	28	Sun		
	29	Mon	Collecting leafminers on Mt Izumi-Katsuragi	
	30	Tue		
	31	Wed	Visit Entomological Laboratory of Osaka Prefecture University	
Sep.	1	Thu	Visit to Natural History Museum of Osaka City	
	2	Fri	Make preparations of leafminers in NWU	
	3	Sat		
	4	Sun		
	5	Mon	Learn taxonomy of leafminers in NWU	
	6	Tue	Move to Hakata, Fukuoka Prefecture	
	7	Wed	Visit Entomological Laboratory of Kyushu University, and learn taxonomy of leafminingDiptera and Lepidoptera	Under supervision by Dr Abe and Hirowatari
	8	Thu		
	9	Fri	Travel back to Nara	
	10	Sat		
	11	Sun		
	12	Mon	Visit Agricultural Exp. Station of Nara Prefecture in Ohwada City to learn about protection of crops from leafminers.	Under supervision by Dr Takenaka
	13	Tues		
	14	Wed	Learn taxonomy of leafminers in NWU	
	15	Thu		
	16	Fri		
	17	Sat		
	18	Sun		
	19	Mon	Day off	National Holiday
	20	Tue	Learn taxonomy of leafminers in NWU	
	21	Wed	Travel to Sapporo, Hokkaido Prefecture	
	22	Thu	Collect leafminers in the forest of Ishikari Coast	Under supervision by Dr Kimura
	23	Fri	Visit Natural History Museum of Hokkaido University.	
	24	Sat	Identify the species of leafminers in Hokkaido University	
	25	Sun		
	26	Mon	Visit Apple Research Institute of Aomori Prefecture to learn about apple leafminers	Under supervision by Dr Ishiguri
	27	Tue		
	28	Wed	Return to Nara	
	29	Thu	Organize data and specimens, and final discussions in NUW	
	30	Fri	Return to Thailand	

4. Daily Activities (Laboratory Studies)

A) Laboratory Studies

4.1 Preparation of genitalia slides of leafminers

Professor Sato provided instruction in the preparation of slide mounts of the genitalia of leafminers because the characteristics of genitalia are very important for leafminer identification. Preparation of genitalia slides was conducted at Nara Woman's University.

Materials and methods (Figure 1)

1) Leafminer larvae and pupae were obtained from many kinds of trees from the forest. Adults were reared from larvae and pupae collected in the field.
2) Genitalia were dissected, stained with 2% fuchsin (in a mixed solution of lactic acid and phenol in the ratio of 3 to 1) for 2 hours at 70°C.
3) Genitalia were also stained with chlorazol black (in 70% ethanol)
4) Genitalia were mounted in Canada balsam or Euparal. Male genitalia were mounted with widely spread valvae, so that the inner surfaces faced upwards. Female genitalia were left attached to the abdominal segments VII and VIII, and were mounted ventral side up.
5) Species were identified from adults emerging from mines and/or by their pupal cremaster.

Laboratory work in Nara also included extensive practice in the identification of leafminers belonging the families Gracillaridae, genera *Phyllonorycter*

4.2 Molecular study

I visited the laboratory of Associate Professor Dr Issei Oshima, Department of Life and Environmental Sciences, Kyoto Prefectural University. His research investigates the

process and mechanism of the evolution of complex traits. His focus is on host shifting events in phytophagous insects, which require evolution of adult female preferences and larval performance. He is interested especially in the genetic basis of ovipositing female preferences and larval performance. His current work involves QTL mapping of the genes responsible for host adaptation using the leaf-mining moth *Acrocercops transecta* (family Gracillariidae) as a model system. I spent three days with Dr Oshima to learn about DNA sequencing techniques (Figure 2 –7). I learnt how to perform DNA extraction, polymerase chain reaction (PCR) amplification, sequencing and purification as detailed below.

Molecular procedure and analysis

- Mitochondrial DNA extraction

The abdomen was removed from each individual for genomic DNA extraction using the ISOLATE II Genomic DNA Kit (Qiagen) following the manufacturer's protocol. A 650 bp fragment of mitochondrial cytochrome c oxidase subunit I (*cox1*) was amplified using the universal invertebrate *cox1* primers:

LCO1490 (forward: GGTCAACAAATCATAAAGATATTGG) and

HCO2198 (reverse: TAAACTTCAGGGTGACCAAAAAATCA) (Folmer et al., 1994;

Wilson, 2012).

-Polymerase chain reaction (PCR) amplification sequencing and Sanger sequencing

PCR amplification was carried out with 1 µl of template DNA, 2µl of each primer (10 pmol/µl), 2 µl of 10X polymerase buffer, 1.6µl of dNTP, and made up to a final volume of20µl with distilled deionized water (ACMG).

Amplifications were performed in a thermal cycler with an initial denaturing step at 94˚C for 3 minutes followed by 28 cycles at 94˚C for 30 seconds, 50˚C for 30 seconds, 72˚C for 30 seconds, and a final elongation step at 72˚C for 5 minutes.

- Gel electrophoresis

One µl of PCR product was separated in 1.5% (w/v) agarose gel using TBE buffer (40 mMTris acetate, 1 mM EDTA) to confirm the quality of PCR product.

-PCR Purification

Total PCR product was purified using the commercial ISOLATE PCR and Gel Kit following the manufacturer's guidelines. Purified PCR product was amplified in a sequencing reaction containing 1.0 µl of PCR product, 1.0 µl of forward primer (3.2pmol/µl), 0.5 µl of version 3.1 ABI Prism® Big Dye Terminators(Applied Biosystems, California, USA), 3.5 µL of 5x sequencing dilution buffer(400mM Tris pH9, 10mM MgCl2), adjusted to a total reaction volume of 20µL withddH2O (double distilled water). Purified PCR were sent to sequencing at the Molecular Genetics Company in Japan.

- Mitochondrial DNA analyses

1) Sequences were aligned by eye and checked for internal stop codons and double peaks (indicative of pseudogenes) using FOURPEAK Sequence Alignment Editor for MAC and BioEdit Sequence Alignment Editor Version 7.2.5 (Hall, 1999) for WINDOW.

2) Analysis of genetic diversity (number of haplotypes) was performed using MEGA version7.0 (Tamura et al., 2007).

3) Median joining networks among leafminers from Nara and Hokkaido haplotypes were constructed and post-processed under maximum parsimony in Network Version 4.6.1.1 (Bandelt et al., 1999). Median joining networks were considered the most appropriated method in this case over other alternatives (e.g., minimum spanning and maximum parsimony). Minimum spanning networks perform best when sampling of haplotypes across the population is relatively complete; they do not perform well if there

are significant gaps in sampling across the distribution of a species and where some internal node haplotypes are not sampled. On the other, hand, median joining networks incorporate the maximum parsimony criterion and infer internal node haplotypes that may have been missed by incomplete sampling (Cassens et al., 2005).

B) Field Studies

Leafminers were collected from the field and returned to the laboratory where larvae completed development. Adults were reared from those larvae and pupae that were collected in the field. The targeted leafminers depended on availability, but the nominated hosts were species that are typically attacked by Lepidopteran leafminer.

In total, I collected leafminers at five locations in Japan. Specimens were obtained from Nara Hill, Yata Hill, Takamado Hill, Mt Izumi -Katsuragi and Ishikari Coast.

Field collection

1) Nara Hills

Leafminers were collectedfrom the hills of Nara City on the 2 August 2016 (Figures 8). The vegetation of the hills is bamboo predominantly deciduous woodland with evergreen pines. Many species of leafminers were found and taken back to the laboratory.

2) Yata Hill

Leafminers were collected in Yata hills on 22 August 2016 (Figure 9 and 10). The Yata hills are part of Yata Prefectural Natural Park to the northwest of Nara. The vegetation is / similar to Nara Hills. Temples in the park include Ryōsenji, Yatadera, Tōmyō-ji, and Matsuodera. The Yata hills run from north to south for about 10 km. Many species of leafminers were found and taken back to the laboratory**.**

3) Takamado Hill

We carried out the leafminer collecting in a red pine (*Pinusdensiflora* Siebold & Zuccarini, Family Pinaceae) forest on Takamado Hill near the urban area of Nara on 22 August 2016 (Figure 11). The understory vegetation of the forest was dominated by *E. japonica*. Many species of leafminers were found and taken back to the laboratory.

4) Mt Izumi-Katsuragi

Mount Izumi-Katsuragi is a mountain in the Kongō Range, the border between Osaka and Wakayama Prefectures in Japan. Its peak elevation is 858 metres (Figure 12). The north slope of Mount Izumi-Katsuragi is covered in a wood of Japanese beech. This species is fast growing here because the elevations are high enough to remain fairly cool. This is the southernmost stand of Japanese beech on Honshū. The trees in the area were designated as a nationally protected species in 1923, which has allowed them to continue to thrive under suburban and urban growth. Unfortunately, our visit coincided with a typhoon and we were unable to collect as many leafminers as we had hoped.

5) Ishikari coast, Hokkaido

We also collected leafminers from the Ishikari coast. Many specimens were collected from oak leaves from eight locations along the coast. After collecting we put those mines in boxes and Dr. Sato sent them back to Nara (Figure 13).

C) <u>Specimen Collection Study</u>

1) Visit to Entomological Laboratory of Osaka Prefecture University

We visited the insect collection at the Entomological Laboratory of Osaka Prefecture University (Figure 14). There are many kinds of insects in this collection, including butterflies and moths from Thailand. Professor Dr Yutaka Yoshiyasu, who is an expert on microlepidoptera and also the curator, showed us the insect collection and gave

me valuable reference books for the library associated with the Thai Department of Agriculture insect collection (Microlepidoptera of Thailand: scientific results of the Lepidoptera ecological Expeditions of the University of Osaka Prefecture to Thailand). These books will be very useful because there are many Lepidoptera specimens in the Department's collection that have not yet been identified. I will be able to use these books as a reference for future identification.

2) Natural History Museum of Osaka City

We also visited the Natural History Museum of Osaka city (Figure 15). The collections of recent insect sand other terrestrial arthropods, including spiders, comprise an estimated 700,000 prepared specimens. The number of specimens is increasing by more than 10,000 per year due to the cooperation of many people and students. Most of them are dried specimens except for the larvae as specimens with alcohol and minute insects. The collection also includes paratype specimens. The total number of specimens amounted to 649,330 in 2001. Approximately 80% of the specimens were collected from Japan and 20% from other countries.

D) Other activities

1) Visiting Entomological Laboratory of Kyushu University.

We were hosted in Kyushu by Dr Yoshihisa Abe, Professor-Entomology, Faculty of Social and Cultural Studies and his Ph.D. student Mr Taguchi Daisuke, Graduate school of Integrated Sciences for Global Society. Dr Abe is interested in leafminer parasitoids. He gave us a presentation about the different displacement directions in two species of exotics and Mr Taguchi Daisuke also presented about mode of parasitism in *Gronotoma micromorpha* (Hymenoptera; Eucoilidae).

In this laboratory, we also learnt about the taxonomy of leafmining Diptera using male genitalia as per the protocol below.

Genitalia preparation: The males were examined for identification.

- The complete abdomen of each individual was removed and placed in 5 ml of 10% potassium hydroxide (KOH) solution
- Boiled at temperature (70 °C); this process softened and cleared the structures for ease of dissection.
- Following softening, abdomens were dissected under water.
- Each aedeagus was removed from the remaining genitalic structures and straightened on a microscope slide.

In addition, we leant how to rear the dipteran leafminer and its parasites. Parasitic wasps (parasitoids) of the family Eulcoilidae (order Hymenoptera) are important in natural control and, in the absence of insecticides, usually keep this insect at low levels of abundance. Two parasitoid species are known from Kyushu University. Species of Eulocoilidae such as *Gronotoma micromorpha* (Perkins) are generally found to be most important (Figure 16 -17).

Also at Kyushu University, Professor Dr. Toshiya Hirowatari, Entomological Laboratory, Faculty of Agriculture has been working on the taxonomy and phylogenetic systematics of insects, especially on moths and butterflies (Lepidoptera including leafminers). He showed us the Insect exhibition (Figure 18) and taught us how to identify moths to family level and identified specimens from insect collection (Figure 19).

2) Agriculture Research and Development Center, Agricultural Experiment Station of Nara Prefecture in Ohwuda City.

In this research station, we learnt about the protection of crops from leafminers from researchers (Mr Takeo Imura, Chief Researcher and Mr Yoshinori Kunimoto). They showed us the damage from leafminers and the tomato plantations using pesticide and non-insecticide (Figure 20). We also had a chance to visit the insect collection in which

mostly contains leafminers (Diptera and Lepdioptera) from tomato (Figure 21). We surveyed leafminer in onion plantation and found the larvae of the dipteran leafminer (*Liriomyza achiensis*) (Figure 22). We also visited another Experiment station (a branch of Agriculture Research and Development Center, Agricultural Experiment Station of Nara Prefecture) and another Experiment station and famer's plantations in that area (Figures 23, 24).

3) Kashihara City Museum of Insect, National Museum of Nature and Science.

We had the opportunity to visit the insect museum in Kashihara city. Mr. Fumiaki Kimura showed us the butterfly greenhouse and how to mass rear insects for exhibition (Figure 25). The Kashihara City Insectarium is especially interesting because the museum room is exhibited as a history of life and as insects' physical characteristics. In the Ecological Exhibit Room there is a device with allows you to observe insects with a miniature camera from an insect's perspective (Figure 26).

4) Aomori Prefectural Industrial Technology Research Center

The Apple Research Institute of the Aomori Prefectural Industrial Technology Research Center is located in Kuroishi city and is the only apple experimental research institution in Japan. The Apple Research Institute was established in 1931 and carries out experimental research on apples and other fruit trees. In this institute under supervised Dr Ishiguri, we surveyed the apple orchard and collected mines to be taken back to the laboratory. Four species of leafminer and their parasitoids have been found. We also visited the Apples Archives Building which serves as a historical museum displaying information about apple cultivars and history.

5) Summary of the Attachment

The attachment program on diagnostics of leafminer of agriculture importance took place from 1 August to 30 September 2016. Two participants from Thailand and Singapore were nominated. Because I have been working in the Insect Taxonomy group which is also responsible for the principle insect museum of Thailand, the program in Japan has been very useful for my day-to-day work and long-term responsibilities. I have learnt many things from this attachment program both with regard to the general management and display of reference collections and the identification of leafmining pests.

I have not only learnt how to prepare and identify leafminers (both Lepidopteran and Dipteran leafminers) but also gained knowledge about collecting and mass rearing of these pests. The taxonomic training encompassed family, genus and species level identification and was greatly aided by having extensive authoritatively identified specimens, up-to-date literature and the guidance of experienced taxonomists. I am grateful that the attachment has enable me to establish good working relationships with several specialists with whom I can consult in the future. The practical nature of the work, in which specimens were collected from the field or reared, prepared and then examined, was especially welcome.

Moreover, we have visited much Insect collection throughout Japan such as Osaka Natural museum, the Insect museum in Nara and the Hokkaido museum. That has been a good opportunity for me because I have gained more ideas and knowledge on how to improve my Insect museum in Thailand, both as a reference collection and as a teaching resource.

The most important thing is I have had a chance to learn how some of the most important agricultural products of Japan (vegetable and apple) are grown and how pest problems are managed. Many of these practices are widely applicable. I have a unique

opportunity to learn numerous and diverse techniques from many experienced Professors and specialists. Everything that I have learnt from Japan can be applied to Thai agricultural product, either in principle or practice.

6) Recommendation for Future Activities

Based on my experience of this attachment program, I would like to recommend that for future activities:

- The duration of two months is not sufficient for optimal learning. For challenging and diverse groups such as leafminers at least two months is required to learn how to identify genera and species. This is in addition to the time required to become confident in the specialist techniques necessary for collecting, rearing and preparation of specimens.

7) Acknowledgements

I wish to express my sincere appreciation and profound gratitude to Professor Dr Hiroaki Sato, Nara Women University for his invaluable guidance, constructive comments and kind encouragement throughout my study. As my primary advisor, he helped me with my every need. I extend my sincere appreciation and heartfelt gratitude also to the other lecturers, Associate Professor Dr Issei Oshima, Osaka Prefecture University, Professor Dr Yoshihisa Abe, Entomology, Faculty of Social and Cultural Studies and Professor DrToshiya Hirowatari, Entomological Laboratory, Faculty of Agriculture, Kyusu University, Mr Takeo Imura, Mr Yoshinori Kunimoto and his team from Agriculture Research and Development Center, Agricultural Experiment Station of Nara Prefecture in Ohwuda City, Kashihara City Museum of Insect, National Museum of Nature and Science in Kashihara city for their hospitality, crucial advice and suggestions during the study period.

I am profoundly indebted to Dr Lum Keng Yeang and Dr Soetikno S. Sastroutomo, APHCN-ASEANET for their encouragement, valuable comments and suggestions.

Finally, I would like to express my gratitude to the Japan ASEAN Integration Fund (JAIF). This attachment program would not have been possible without the support of JAIF that have provided financial to keep this project running.

8) References

Bandelt, H. J., Forster, P. & Röhl, A. (1999). Median-joining networks for inferring intraspecific phylogenies. *Molecular Biology and Evolution, 16*(1), 37-48.

Cassens, I., Mardulyn, P., & Milinkovitch, M. C. (2005). Evaluating intraspecific "network" construction methods using simulated sequence data: do existing algorithms outperform the global maximum parsimony approach?. *Systematic Biology, 54*(3), 363-372.

Hall, T. A. (1999). BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *In Nucleic Acids Symposium Series, 41*, 95-98.

Tamura, K., Dudley, J., Nei, M., & Kumar, S. (2007). MEGA4: Molecular Evolutionary Genetic Analysis (MEGA) software version 4.0. *Molecular Biology and Evolution*, *24*, 1596-1599.

9) Images/Photos

Figure 1 Genitalia slide preparation. Dissected parts were passed through a series of graded alcohols in the small, ceramic pots shown in this photograph prior to mounting.

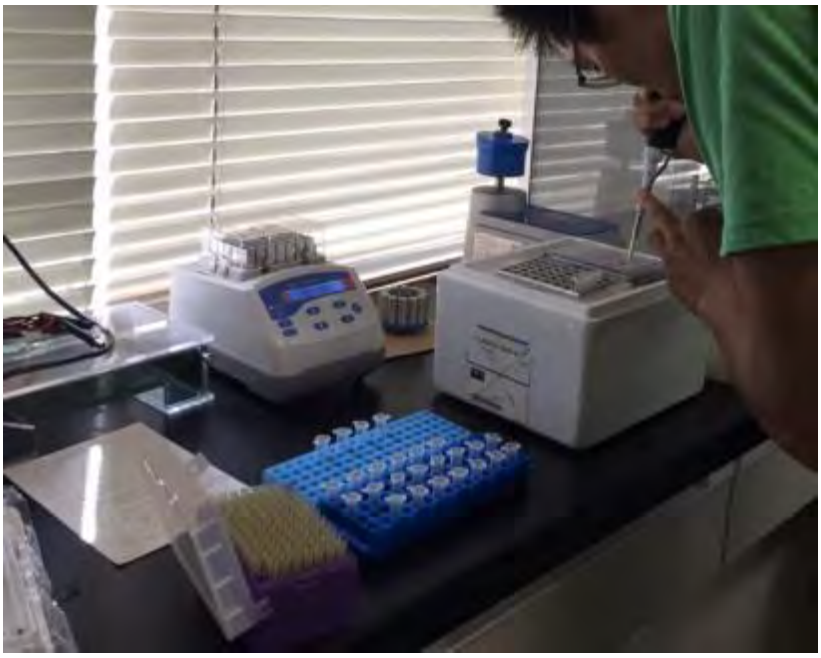

Figure 2 Demonstration how to prepare the PCR reaction by Dr Issei Oshima.

Figure 3 Demonstration of how to obtain information from leafminers sequence by Dr Issei Oshima.

AG-01_COIH-PREMIX.fasta — Edited

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NNNNNNNNNNCTCCTCCTGCAGGANCAAAAAATGATGTATTTAAGTTTCGATCAGTT
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Figure 4 Sequence of leafminer (fasta file).

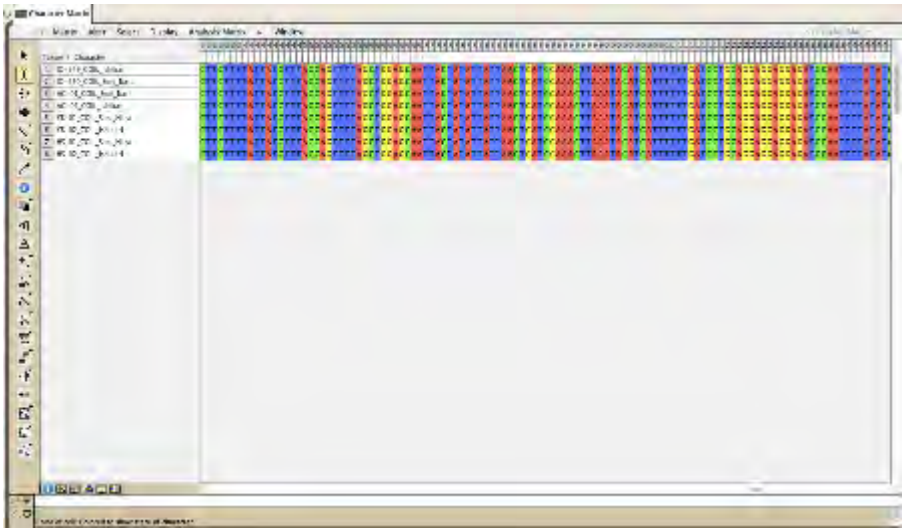

Figure 5 Sequence of leafminer (MEGA file).

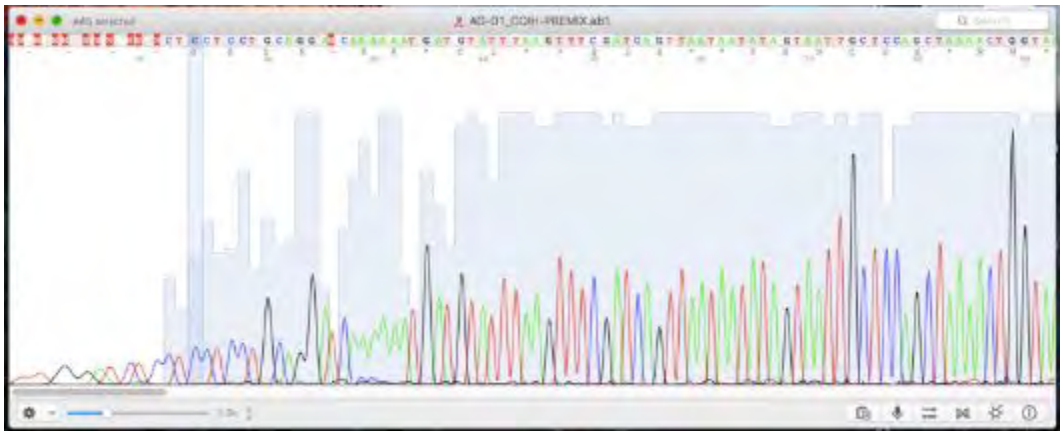

Figure 6 Sequence of leafminer from 4peak program.

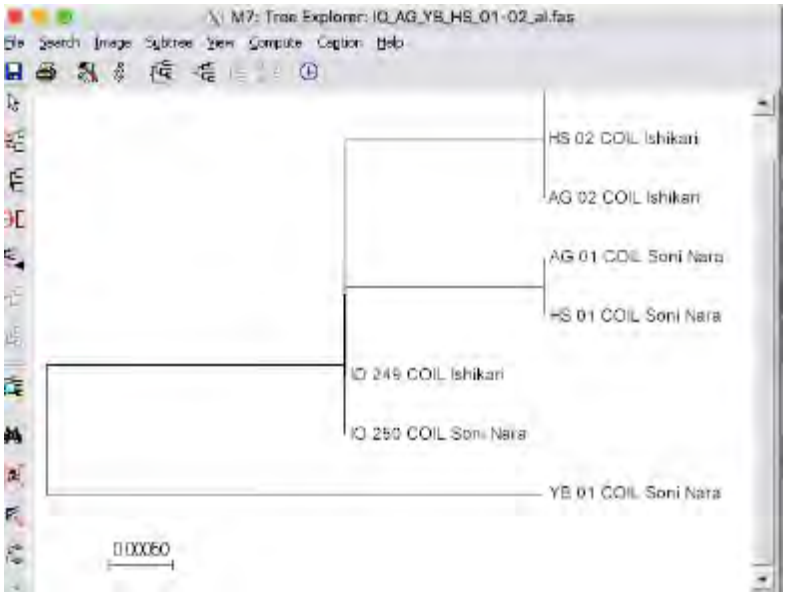

Figure7 The evolutionary history of the sampled leafminers was inferred using the Neighbor-Joining method. The optimal tree with the sum of branch length is shown. The tree is shown the evolutionary distances used to refer the phylogenetics.

Figure 8 Collecting location at Nara Hill.

Figure 9 Collecting location at Yata Hill.

Figure 10 Leafmines at Yata Hill.

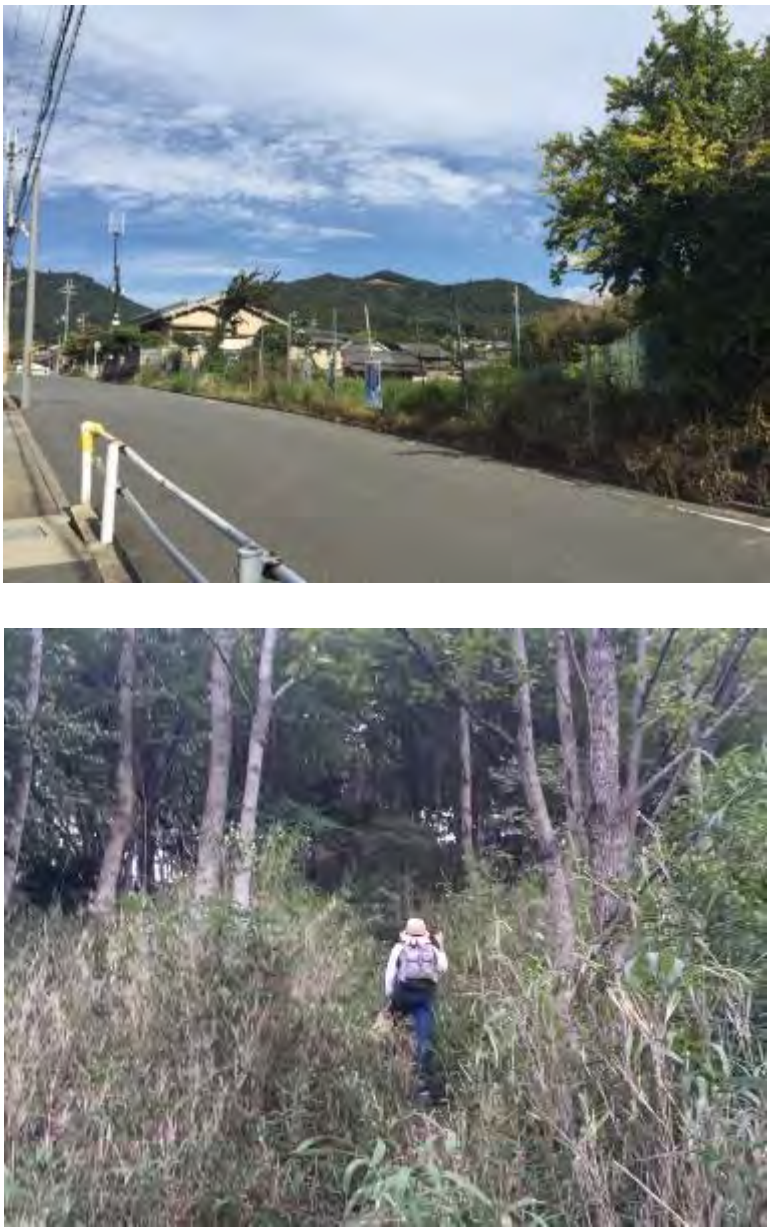

Figure 11 Collecting location at Mt. Takamado.

Figure 12 The collecting location at Mt Izumi-Katsuragi.

Figure 13 Field collection from oak trees along the Ishikari coast and transferring leafminer collections to boxes for dispatch to Nara.

Figure 14 The Insect collection in Osaka Prefecture University.

Figure 15 The Insect collection in Osaka Natural History Museum.

Figure 16 Mass rearing leafminers in laboratory in Kyushu University.

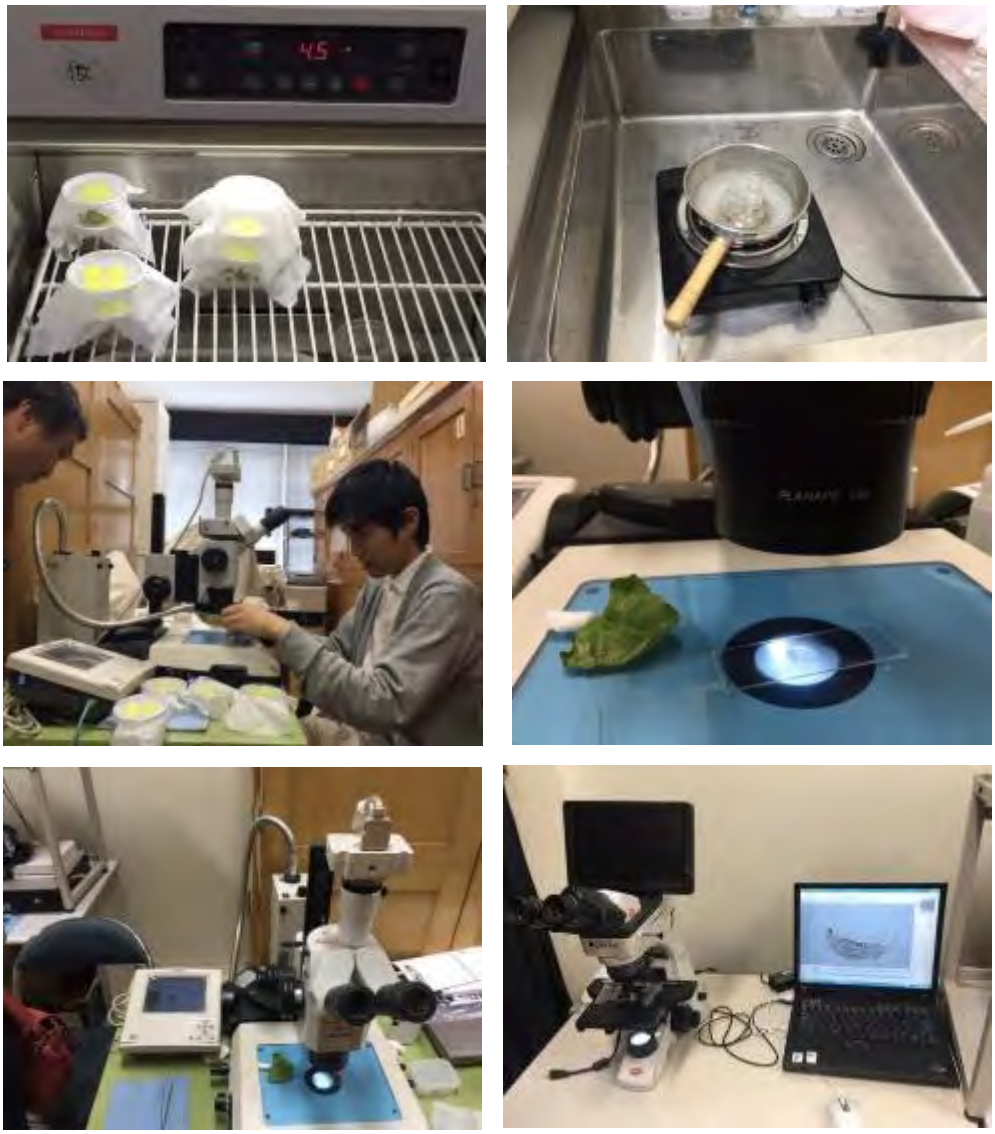

Figure 17 Genitalia study and morphological study of leafminer parasite, Kyushu University.

Figure 18 The Insect ehxibition in Kyushu University.

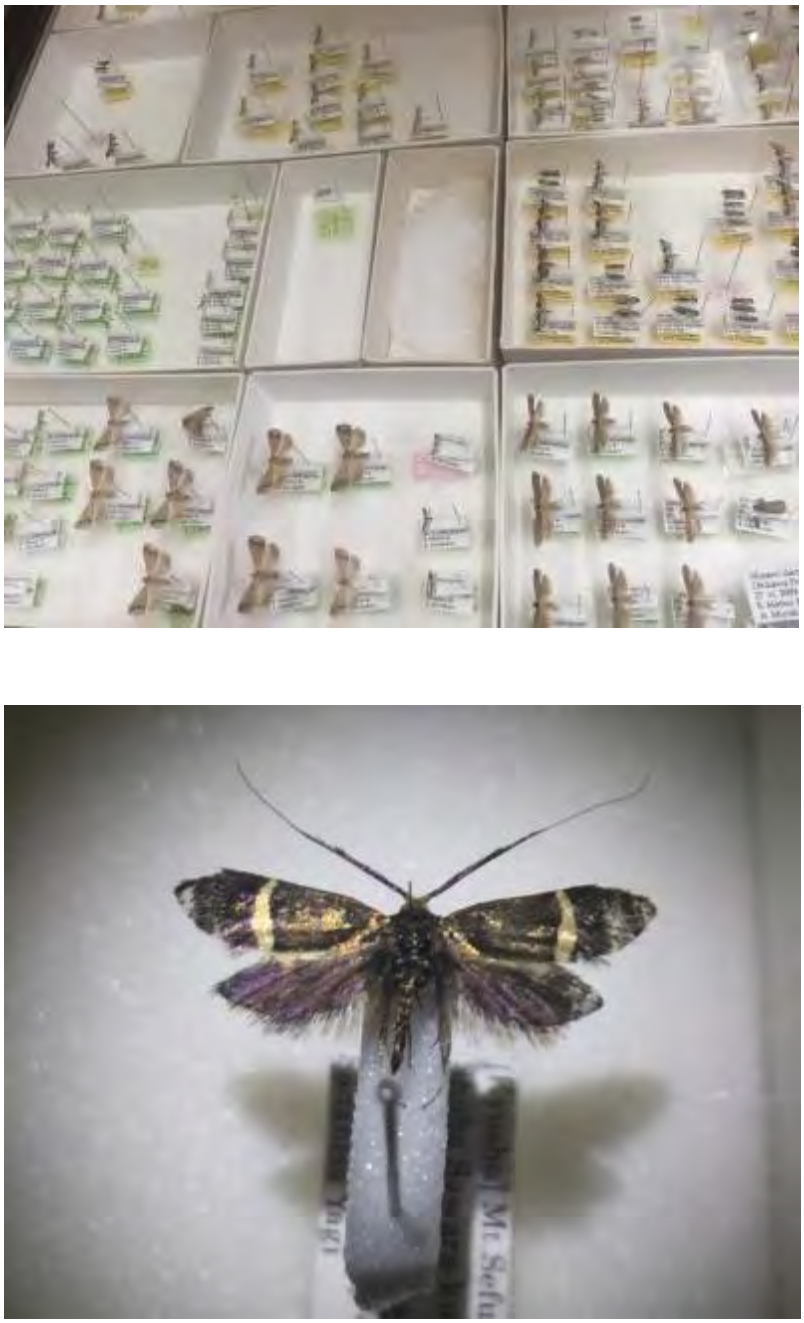

Figure 19 The Insect collection in Kyushu University.

Figure 20 Tomato plantations in Agriculture research and Development Center, Agricultural Exp. Station of Nara Prefecture.

Figure 21 Insect collection in Agriculture research and Development Center, Agricultural Exp. Station of Nara Prefecture.

Figure 22 Onion plantations in Agriculture research and Development Center, Agricultural Exp. Station of Nara Prefecture and leaf mines caused by *Liriomyza achiensis*.

Figure 23 Tomato plantations in Agriculture research and Development Center, Agricultural Exp. Station of Nara Prefecture.

Figure 24 Tomato plantations in the local area.

Figure 25 Insectarium at Kashihara city

Figure 26 The insect exhibition at Kashihara city.

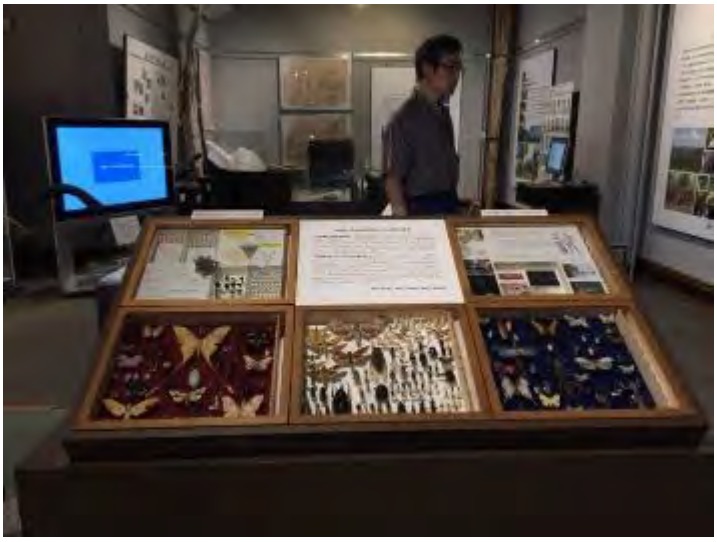

Figure 27 Hokkaido University and Hokkaido university museum.

Figure 28 Leafminer larva and insect pest of apple at Apple Research Institute of the Aomori Prefectural Industrial Technology Research Center, Kuroishi city.