

Field and laboratory manuals for biodiversity survey in the protected areas in Sarawak

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Preface

A project of SATREPS (JST-JICA), entitled "Project on Development of Management Systems for Multiple Utilization of Biodiversity in the Tropical Rainforests at the Protected Areas in Sarawak" (PUBS) led by Takao Itioka was launched in 2019. This research aimed to comprehensively investigate the distribution areas and conservation status of diverse organisms in the tropical rainforests within totally protected areas scattered throughout Sarawak with Next Generation Sequencing (NGS), an advanced technologies using Next Generation Sequencer (also abbreviated as NGS). This project also aimed to construct data archives of biodiversity information and data, based on the results obtained through the research activities implemented in this project. Furthermore, this project aimed to develop data platforms of such data archives that would promote the dissemination of knowledge and information about biodiversity there, cultivate human resources responsible for scientific research and science education in Sarawak society, and contribute to the development of management system aimed at biodiversity conservation in protected areas, that of industries that utilize genetic resources and that of tourism industry that uses intellectual discoveries about biodiversity as content.

Under PUBS, a type of inclusive field survey for unveiling the entire biodiversity in the protected area, which is one of the key activities of the project, called SBPS (Survey for Biodiversity in the Protected areas in Sarawak), was implemented. In the SBPS, different types of surveys targeting various taxa in a wide range of organisms, including insects, fungi, plants, and vertebrates, were conducted in the field at more than 15 protected areas all over Sarawak in collaboration with Sarawakian and Japanese researchers from different academic backgrounds.

In parallel with the implementation of SBPS, significant progress was made in developing infrastructure and research facilities to strengthen research in biodiversity sciences, which is another key activity of our project, has significantly advanced. Various types of equipment, such as the next-generation DNA sequencers (NGSs), their peripheral devices, deep-freezers, microscopes, and digital microscopes with focus stacking function, that provide powerful support for Sarawakian's research on biodiversity sciences, were introduced to the three research organizations, Forest Department Sarawak (FDS), Sarawak Forestry Corporation (SFC), and Sarawak Biodiversity Centre (SBC), from late July to October 2022.

Here, we publish a collection of manuals for field and laboratory biodiversity surveys that were implemented under PUBS. This article consists of four parts, including 18 manuals for studies in the fields of botany, forest ecology, entomology, mycology, zoology and DNA analyses. We hope that, in research on the biodiversity of tropical rainforests in Sarawak, this manual would help researchers, scientists and technicians collect samples and data in the field and conduct molecular experiments to assess the biodiversity.

Acknowledgements

The JICA part of PUBS was conducted for five years (launched in 2021 February and ended in 2026 February) in accordance with the Memorandum of Understanding (MOU) signed by FDS and the Japan Research Consortium for Tropical Forests in Sarawak (JRCTS) in November 2012, with the MOU signed by SFC and JRCTS in February 2021 (renewed in November 2023), and with Record of Discussion signed between

the Sarawak Government and JICA. We thank Datu Sr. Zaidi Bin Haji Mahdi, Datu Haji Abdullah Bin Julaihi, Hajah Hairani binti Haji Mohamad Ismail, Tessy Nimos (Ministry of Natural Resources and Urban Development Sarawak), Datu Haji Hamden Bin Haji Mohammad, Jack Liam, Datin Runi Anak Sylvester Punga (FDS), Haji Zolkipli Mohamad Aton, Abang Arabi Bin Abang Aimran, Oswald Braken Tisen, Melvin Terry Gumal, Haji Madhan Bin Kiflie, Japanie Bin Bujang, Mohamad Kasyfullah Bin Zaini, Mohamad Faraddy Bin Maseri, Abang Norizan Bin Abang Median, Paschal Anak Dagang, James Anak Bali, Nurzehan Binti Ramji (SFC), Dato Dr Yeo Tiong Chia and Margarita Naming (SBC) for approving and supporting our research activities. I would like to express my gratitude to Paulus Meleng, Khairunnisa Binti Othman, Shirley Anak Chip, Princilla Anak Leong (FDS), Clement Het Kalias, Voon Mufeng, Muliati Musa, Jacqueline Anak Henry Ripan, Gleecia Barry, Suzila Kaja Anak Aliang (SFC), Holed Juboi, Jamilah Hassan, Ajuwin Lain (SBC), Asano Iku, Seiya Okuno, Takeru Kawaratani, Akira Itoh and all other Japanese participants of SBPS for participating in the project. We also thank all other research staff who joined and assisted our tasks as project missions in FDS, SFC and SBC, and the staff of national parks where SBPS surveys were conducted for supporting our field and laboratory surveys. PUBS was financially supported by the JST/JICA and SATREPS grants (JPMJSA1902).

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I. Plants/Vegetation

1. A standardized belt transect survey method

S. Tagane and N. Komada

Introduction

There are several ways to record the local flora, including permanent plots and conventional botanical inventories. In this study, we employed a standardized belt transect method (Yahara et al. 2012, Zhang et al. 2016, Toyama et al. 2022). The essential feature of this method is the comprehensive collection of occurrence records of vascular plant species in the surveyed area, along with their voucher specimens, photographs taken in the field, and silica-dried leaf fragments for molecular analysis. These data and materials enabled us to easily recognize and compare plant species diversity among plots placed in various environments in the region. Previous guidelines can be referred for collecting voucher specimens and leaf fragments in the field (Bridson & Forman 1999, Funk et al. 2017).

Field survey

1) A rectangular plot ($5\text{ m} \times 100\text{ m}$) was established and divided into 10 sub-plots ($5\text{ m} \times 10\text{ m}$) (Fig. 1). The GPS coordinates were set for the start and end points of the plot. Because plant species usually have species-specific habitats, the plots may be better placed in different environments and elevations in the region, as well as in various topographies, such as small valleys, slopes, and ridges, to cover as many plant species as possible.

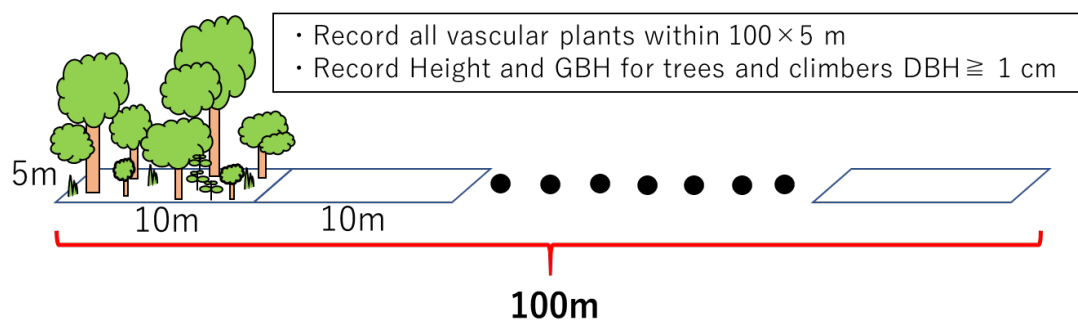


Fig. 1 Concept of a standardized belt transect survey.

2) All vascular plant species, including trees, shrubs, bamboos, palms, climbers, herbs, and epiphytes, were recorded within the first $10 \times 5\text{ m}$. For each species, photographs in the field, voucher specimens and silica-dried leaf fragments (for molecular analysis) were taken when they appeared for the first time. If the exact species/local names in the field are unknown, they can be recorded using tentative field names. In this survey, the most important thing is “discrimination of plant species” to accurately know the plant species diversity, and the ‘correct’ names can be provided after examining the identity of the species using their voucher specimens.

3) Plant height and girth at breast height (GBH) for all trunks/stems of trees and climbers having diameter at breast height (DBH) $\geq 1\text{ cm}$ (GBH $\geq 3.14\text{ cm}$) were recorded. Plant height was measured with the eye (an estimate) or using a measuring pole. GBH were measured using measuring tape.

4) Steps 2) and 3) were followed in the subsequent subplots. We gathered the occurrence records for all species, but the voucher specimens of the species collected in the previous subplots were no longer collected. If one is unsure whether the plant species have already been collected, it is better to collect them. Subsequently the specimens were compared and cleaned.

Products obtained from the survey

Herbarium specimens

Voucher specimens were important for identifying plant taxa and ensuring the reliability of this study. They were pressed, dried, and mounted on paper with a label that provided information about where they were collected from, and stored in the herbarium. Two or three sets of duplicate specimens were prepared for distribution to the other herbaria. Information, including locality (Country, State/Province, City, District, etc.), geo-reference (Latitude, Longitude, Elevation), Date, Collector(s), was required for each specimen. Field notes (flower color, abundance, etc.) must also be filled. Labels for the herbarium specimens were readily produced from this list. For the spreadsheet format, The Darwin Core Standard (DwC) (see Wiecek et al. (2012) for the detail), developed by the Biodiversity Information Standards (TDWG) community (<https://www.tdwg.org/>) is strongly recommended. This will help to share, use, and reuse open-access biodiversity data (e.g., GBIF) in the future.

Simple photo guide

Before making specimens, take photos of (1) whole plant for small herbs, leafy branches (when sterile), or flowering/fruiting branches for trees, (2) abaxial leaf surface, (3) the other characters important for the classification such as flowers, fruits, stipules, terminal buds, outer/inner barks, etc. These photos were then pasted onto a Microsoft PowerPoint slide for each sample (Fig. 2), with their scientific name/tentative field names/local names, sample number, locality, and some notes. These slides were helpful for remembering the plants by family and genus that were collected and sharing them with others.

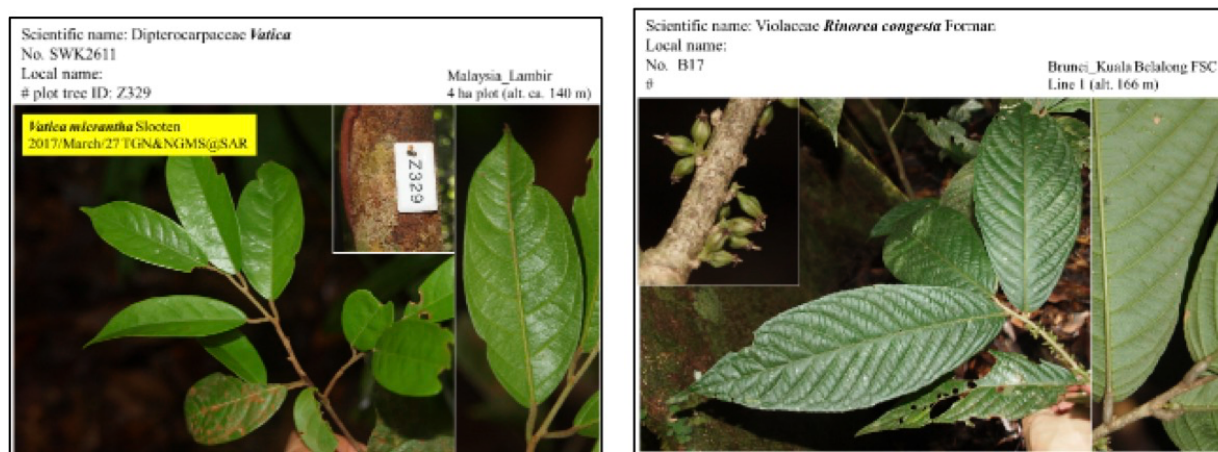


Fig. 2 Examples of photos arranged on the slides of Microsoft PowerPoint.

Dried leaf fragments for DNA analysis

Drying leaf fragments with silica gel in polyethylene bags (Fig. 3) is a standard field-sampling method suitable for subsequent molecular analyses. When plants and press specimens were collected, leaf fragments were taken from each sample and placed individually in tea bags or small paper envelopes. These samples were then placed in plastic bags containing silica gel. Because the quality of DNA depends on time, it is important to dry the leaf fragments as soon as possible. Silica dried samples are stored at -80°C in freezers in the laboratory.



Fig. 3 Dried leaf fragments.

Summarizing data

Presence/absence data for all vascular plant species in a plot

The following data were entered into an Excel spreadsheet as recorded in the field: specimen number, duplicate specimen number, family, field name, GBH, height, and presence (1) in each subplot (Fig. 4). Occurrence data for the same species were then merged into one row (Fig. 5). If we use a consistent tentative name for each species, the results were easily obtained using the pivot table function in Excel.

	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q	R
1	Sort no.	Specimen	Dup. no.	Family	Field name	GBH	Height	0-10	10-20	20-30	30-40	40-50	50-60	60-70	70-80	80-90	90-100m	
2	4	Line 1																
3	5	Start: GPS350	(AA.AAAA°N, BBB.BBBB°E, alt. CCC m)															
4	6	Goal: GPS361	(DD.DDDD°N, EE.EEEE°E, alt. FFF m)															
5	7	4606		Rubiaceae	Mitregyne	89	20	1										
6	8	4607		Pentaphyla	Eurya acuminata	32.5	8	1										
7	9	4608		Apocynaceae	Alstonia	19	5.5	1										
8	10		4607	Pentaphyla	Eurya acuminata	12.3	6	1										
9	11	4643		Casuarinaceae	Casuarina	62	18	1										
10	12		4607	Pentaphyla	Eurya acuminata	23.6	9	1										
11	13		4606	Rubiaceae	Mitregyne	88.5	23	1										
12	60		4643	Casuarinaceae	Casuarina	62.8	18	1										
13	61	4644		Melastomaceae	Bredia	21	4.5	1										
14	62	4645		Urticaceae	Dendrocnide	13.5	4	1										
15	63		4607	Pentaphyla	Eurya acuminata	87.6	17	1										
16	64		4643	Casuarinaceae	Casuarina	70.3	14	1										
17	65	4648		Aristolochiaceae	Aristolochia	1	15	1										
18	66	4646		Myrtaceae	Syzygium claviflorum			1										
19	67	4647		Moraceae	Ficus [long lvs]			1										
20	68	4649		Zingiberaceae	Alpinia			1										

Fig. 4 An example of Excel sheet for data input.

	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O
1	Sort no.	Specimen	Dup. no.	Family	Field name	0-10	10-20	20-30	30-40	40-50	50-60	60-70	70-80	80-90-100m	
2	188	4742		Achariaceae	Rypalosa			1						1	
3	407	4813		Achariaceae	Rypalosa コバ										
4	414	4818		Actinidiaceae	Saurauia									1	
5	138	4709		Anacardiaceae	Semecarpus		1					1			
6	469	4836		Anisophylleaceae	Anisophyllea 大										1
7	265	4773		Anisophylleaceae	Anisophyllea small				1					1	
8	91	4672		Annonaceae	Friesodielsia	1	1								
9	160	4723		Annonaceae	Friesodielsia 2		1						1		
10	242	4762		Annonaceae	Goniothalamus エニコバ				1		1				1
11	458		4611	Annonaceae	Goniothalamus ツツウアカミ									1	1
12	484	4840		Cardiopteridaceae	Gonocaryum minus										1
13	326	4791		Annonaceae	Orophea						1				
14	461	4833		Annonaceae	Popowia?									1	
15	246	4767		Apocynaceae	Aeschinanthus				1						
16	9	4608		Apocynaceae	Alstonia	1									
17	286	4781		Apocynaceae	Apocyna コバ 1 cm					1	1		1		
18	324	4790		Araceae	Aglaonema						1	1			

Fig. 5 An example of Excel sheet after summarizing the data from Fig. 4.

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2. Protocol for Permanent Sample Plot establishment

B. Diway, C. Y. Ling and Y. Takeuchi

1. Permanent sample plots

Permanent Sample Plot (PSP) is a permanently demarcated area, typically 1 ha, in a commercial forest which is established for periodic monitoring (Alder & Synnott 1992). PSPs provide data on how trees grow, which are fundamental to estimate the changes in forest (stocking and volume) over time under different situations, and thus, it is essential for sustainable forest management.

The PSP network in Sarawak has been started in 2016 within the production forest areas. The guideline for the PSP establishment and enumeration is required to ensure systematical establishment and measurement of PSPs following standardized protocol. The guideline considers relevant established references on the subject particularly the former work by Alder & Synnott (1992). The manual of field instruction for the establishment and enumeration of sample plots in inland forest in Sarawak was extensively used and extensively elaborated by Forest Department Sarawak (FDS) (Forest Department Sarawak 2019).

In this section, we will describe a standardized and simplified method for establishing PSPs in concession areas to monitor the long-term impact of logging on forest dynamics, growth, and yields. The data provided estimates of changes in forest stock and volume.

2. Plot establishment/plot demarcation

2.1 Plot location

Plots are established in selected Forest Management Unit areas and natural forests (control plots). Selection of plot location should be guided by a satellite imagery map with GPS coordinates showing forest cover of the areas selected, a topography map of the areas, logging operation maps, and harvesting records for the areas. The selected point locations are then translated to the ground using GPS instruments.

The plot location must be described and tied relative to the nearest fixed base point (tie point), such as permanent topographic features, examples of permanent roads, structures such as Marine Department Markers or Land and survey department telemeter traverse (TT) stations, by bearing and distance, or to absolute geographical coordinates by GPS fixes.

2.2 The plot access line (tie line)

The access line or tie line is a straight line cut along an appropriate bearing from a fixed base point (tie point) to one corner of the plot. The access line should not exceed 5 km; otherwise, relocating the plot is likely to be difficult.

The starting point is marked. The tree closest to the base point is selected, which is the tie tree. Both sides of the tree (approximately 2 m above the ground) are painted in the direction of the access line.

Nail an aluminum marker/signboard for the plot on the tie tree, with the following information (Fig. 1).

PERMANENT SAMPLE PLOT (PSP) Plot Number: GPS Position/distance: Date of Establishment:

Fig. 1 Information signboard for plots to be placed at the tie tree.

The access line is painted blue and white (status quo) as applied to the yield and research plots in Sarawak. This is performed to avoid confusion with block and couple boundaries (Forest Department Sarawak 2019).

2.3 Adjustment to the plot location

A preliminary survey of the plot locations must be conducted before establishing the plot. Adjustment of the plot location is necessary if the following conditions arise:

- Plots fall largely within rivers, swamps, deforested areas, roads, log landings, and other features that make the plots very difficult to survey.
- Large, buttressed trees intersect the plot corners (preliminary surveys should also be conducted along plot boundaries).

The plot can be moved back or forward along the access line bearing in fixed multiples of 100 m to avoid anomalies (Synnott 1979).

2.4 Buffer zones

Buffer zones are demarcated around the plot and established as protection from disturbance or logging. A buffer zone measuring 50 m from the outer boundaries of the plot is recommended, cut, and painted green and white (status quo) (Forest Department Sarawak 2019).

2.5 Establishment of plot layout

In this study, a 100×100 m square plot (1 ha) containing ten 10×10 m square coordinates was set. The first two boundary lines were cut from the end of the access line. The outer boundary lines were cut on a compass bearing at 90° , 180° , and 270° right angles from the end of the access line, as recommended by Synnott (1979). The first corner of the plot at the end of the access line should be marked as A (Fig. 2).

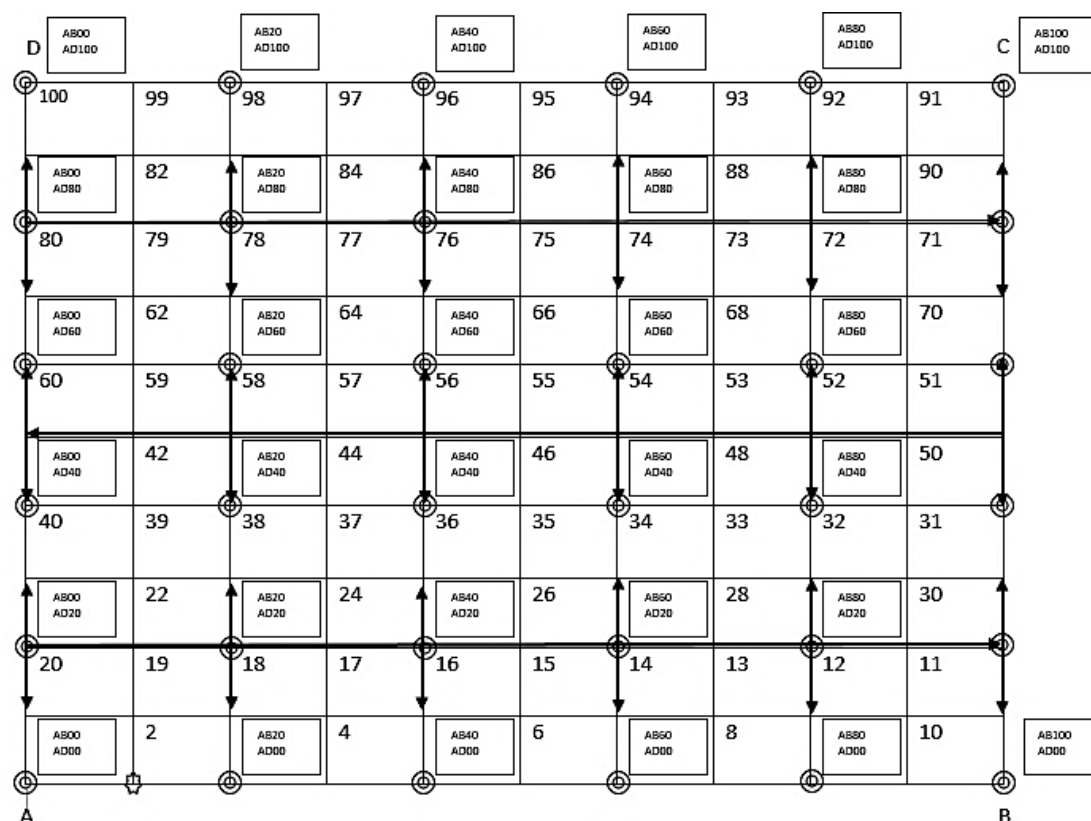


Fig. 2 Marking of plot and quadrates corners.

Plot corners and quadrate boundaries should be marked with 3-meter-long PVC pipes painted red at the top (approximately 1 m long). The plot corners, quadratic boundary intersections, and quadrats were numbered (Fig. 2).

All distance measurements of the plot and quadrat boundary are referred to as horizontal distances. When the terrain is flat, the distance could be directly measured. However, in steep terrain, the slope correction table (Appendix 1) should be used to determine the distance to cover if the slope angle is greater than or equal to 15 percent. With the help of clinometers, measure the slope angle (θ) of AB (Fig. 3). It was ensured that the measurement is taken parallel to the ground slope. For example, the distance correction for a horizontal distance of 20 m with a slope angle of 30% is 20.9 m.

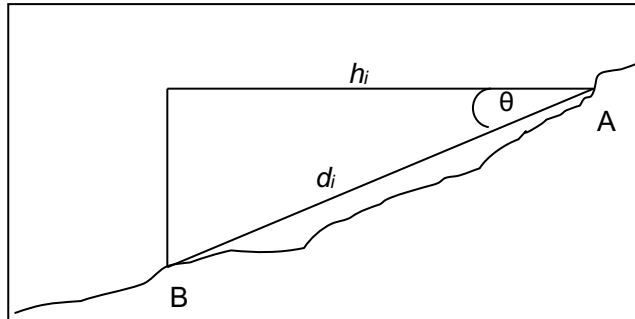


Fig. 3 Measuring slope angle.

Any slashing/clearing of plot boundaries should be done outside the plot. No vegetation should be removed from the plot while establishing or enumerating the sample plot except for the access line. Any stake used for plot establishment should be taken from outside the plot and not closer than 20 m from the outer boundary of any sample plot (Forest Department Sarawak 2019).

2.6 Tree numbering and marking

All trees at 10 cm and above were measured in this study. Metal alloys or aluminum tags that can be punched or engraved should always be used to mark numbers on trees. Corrosion-resistant nails are suitable nailing tags for trees. Nails should be placed 30 cm above or below the point of measurement (Alder & Synnott 1992). If placed close to the Point of Measurement (POM), callus formation around the nail distorts the diameter measurement. Sequential numbers should be used such that each tree has a unique number. A given number should never be reused even if the tree dies or is removed.

3. Plot assessment of trees ≥ 10 cm DBH

All commercial and potentially commercial trees were enumerated, excluding lianas, palms, and rattans, climbers, and non-woody plants.

3.1 Measuring diameter

Minor variations in the methods and procedures of diameter measurement can cause large errors in the growth increment, leading to unreliable data for growth, yield modelling, and volume estimation. The standard measurement procedures and point of measurement (POM) were clearly defined and marked with yellow paint for consistent growth data.

For trees without buttresses, the POM of tree diameter is at 1.3 meter above ground level. A pole marked at 1.3 m was pushed firmly into the leaf litter to the mineral soil next to the tree to define POM (Swaine et al. 1987, Condit 1998). The exact measurement points are marked on the tree. A continuous band was painted around the POM, and the top of the band marks indicated the position of the measuring tape.

For trees with high buttresses (higher than 1.3 m), POM was 30 cm (Fig. 4) above the top of the buttress (Alder & Synnott 1992, Condit 1998).

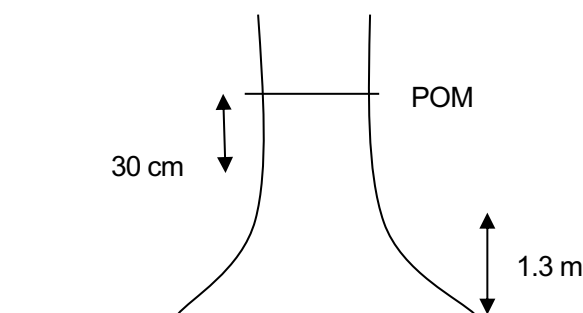


Fig. 4 POM for trees with buttresses.

Deformities/Excrescences: For trees with deformities or excrescences at 1.3 m, the measurement was made above the abnormality (approximately 30 cm above). The recorded height of the POM indicates the presence of an abnormality on the recording form by referring to the three condition codes (Appendix 2).

Forked trees: Trees that forked below or at the measurement point were treated as two trees. Point of measurement for fork below 1.3 m the measurement should be at 1.3 m above ground level. If the fork is 1.3 m above the ground level, the point of measurement should be 30 cm above the ground level (Fig. 5).

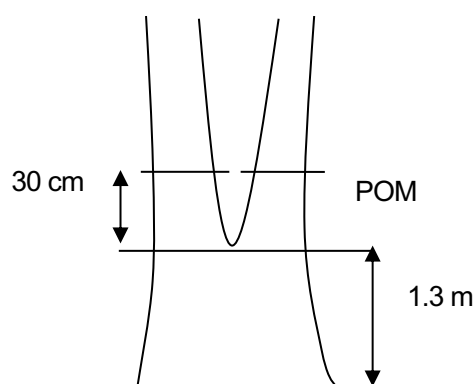


Fig. 5 Point of measurement for forked tree.

Stilt rooted trees: For stilt-rooted trees, the POM should be measured 30 cm above the highest stilt root, and the height of the POM should be recorded.

Slope and fallen or leaning trees: Trees that had fallen or leaned were always measured at a 1.3 m length along the side of the stem closest to the ground (Fig. 6a and 6b).

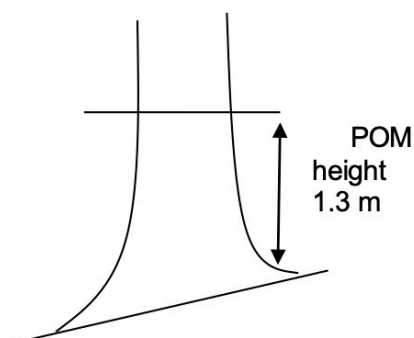


Fig. 6a Trees grow on slope.

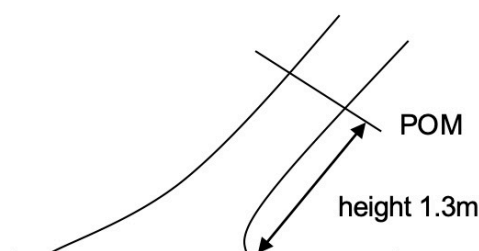


Fig. 6b Leaning or fallen trees.

For the diameter measurement of trees with climbers or lianas, the diameter tape should pass under veins or roots on the stem.

Presence of climbers or stranglers: For trees with climbers, climbers must be removed or cut.

Moving the point of measurement

As the buttresses or stilt roots reached the POM originally adopted, a new POM was established 30 cm above the first. The diameters of both the old and new POM were recorded. It is noteworthy that the POM moved. The old POM was not measured during the next reassessment.

3.2 Height measurement

Tree height was estimated from the ground level to the crown point height and the highest point of the crown. The crown-point height is usually referred to as the height of the first major branch. The measurement should be reduced to the nearest meter means, for example 15.1 m and 15.2 m should be recorded as 15.0 m and 15.3 m, respectively, and 15.4 should be recorded as 15.5 m. Clinometers and distance tapes, or hypsometers, are instruments used to measure height.

3.3 Tree condition/Stem identity class

Field notes on tree conditions are important, because mortality can be associated with causal factors. A coding system for the stem identity class should be adopted for a systematic approach and appropriate statistical analysis. The stem identity class was scored for all living and dead stems. The dead stems of the trees were not recorded during the initial enumeration. The tree condition codes (Appendix 2) provided the stem identity classes used to enumerate the sample plot.

4. Recording field data

The field data recording form was simplified from the field data sheet designated by Forest Department Sarawak (2019) based on silvicultural operation experiments that had been carried out in the logged-over inland forest in Sarawak. This field data form suits the objectives of establishing the 2016 PSP. The details of the field data are provided in Appendix 3, 4.

4.1 Assessment sequence

The assessment sequence should follow the orientation of the quadrat number for easier tracing of the tree-tag sequence in future reassessments.

4.2 Coordinates (X and Y)

The X and Y coordinates indicate the positions of the trees measured in each quarter. The positions ranged from 0 to 10 m for both axes. A value of 0 or 10 m indicated that the position of the tree fell on the quadrat boundary.

5. Assessment of saplings (1 – 9.9 cm) and seedlings

A total of 25 subplot measuring 2×2 m were established at the corners of every $20 \text{ m} \times 20 \text{ m}$ subplot. DBH was measured at a height of 1.3 m, POM was marked, and the trees were numbered. An aluminum wire was used to tie the tree tags.

Seedlings were regarded as having a height of more than 20 cm and a size of not more than 1 cm DBH. The total number of seedlings in every 2×2 m subplot was counted and recorded.

6. Model plan for field survey

10 People for 4 days per plot

Day 1 PLOT ESTABLISHMENT

Day 2 PLOT ASSESSMENT OF TREES ≥ 10 CM DBH

Day 3 PLOT ASSESSMENT OF TREES ≥ 10 CM DBH

Day 4 ASSESSMENT OF SAPLINGS

Acknowledgments

We are grateful to the Forest Department Sarawak and Sarawak Forestry Corporation for their support of our field research in the forests of Sarawak. We also acknowledge Mr. Tan S. for his contributions to establishing the first methodology for forest plot surveys inland Sarawak. This methodology is use for base reference for the establishment and enumeration of permanent forest plots in Sarawak.

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Appendix 1: Slope correction table.

Slope	Degree	Factor	Horizontal distances										Slope
%	°	f_s	5	10	15	20	25	30	40	50	125	245	%
15	9	1.0112	5.1	10.1	15.2	20.2	25.3	30.3	40.4	50.6	126.4	247.7	15
20	11	1.0198	5.1	10.2	15.3	20.4	25.5	30.6	40.8	51.0	127.5	249.9	20
25	14	1.0308	5.2	10.3	15.5	20.6	25.8	30.9	41.2	51.5	128.8	252.5	25
30	17	1.0440	5.2	10.4	15.7	20.9	26.1	31.3	41.8	52.2	130.5	255.8	30
35	19	1.0595	5.3	10.6	15.9	21.2	26.5	31.8	42.4	53.0	132.4	259.6	35
40	22	1.0770	5.4	10.8	16.2	21.5	26.9	32.3	43.1	53.9	134.6	263.9	40
45	24	1.0966	5.5	11.0	16.4	21.9	27.4	32.9	43.9	54.8	137.1	268.7	45
50	27	1.1180	5.6	11.2	16.8	22.4	28.0	33.5	44.7	55.9	139.8	273.9	50
60	31	1.1662	5.8	11.7	17.5	23.3	29.2	35.0	46.6	58.3	145.8	285.7	60
70	35	1.2207	6.1	12.2	18.3	24.4	30.5	36.6	48.8	61.0	152.6	299.1	70
80	39	1.2806	6.4	12.8	19.2	25.6	32.0	38.4	51.2	64.0	160.1	313.8	80
90	42	1.3454	6.7	13.5	20.2	26.9	33.6	40.4	53.8	67.3	168.2	329.6	90
100	45	1.4142	7.1	14.1	21.2	28.3	35.4	42.4	56.6	70.7	176.8	346.5	100
110	48	1.4866	7.4	14.9	22.3	29.7	37.2	44.6	59.5	74.3	185.8	364.2	110
120	50	1.5620	7.8	15.6	23.4	31.2	39.1	46.9	62.5	78.1	195.3	382.7	120
130	52	1.6401	8.2	16.4	24.6	32.8	41.0	49.2	65.6	82.0	205.0	401.8	130
140	54	1.7205	8.6	17.2	25.8	34.4	43.0	51.6	68.8	86.0	215.1	421.5	140
150	56	1.8028	9.0	18.0	27.0	36.1	45.1	54.1	72.1	90.1	225.3	441.7	150

Appendix 2: Tree condition code.

Codes	Condition
BS	Broken stump: Only a broken stump has been found at the tree's coordinates.
BT	Broken top: The crown has completely broken off.
CD	Crown damage: Major branches have been broken in the crown
CI	Climber infestation: Two crown of the tree has been invaded by climbers, which are seriously competing with the trees' own foliage. This code should not be applied if the climber foliage is generally below the main tree crown, and therefore not offering serious competition.
DB	Dead bark: Bark has been lost over part of the stem, exposing wood underneath.
DC	Diameter change: The reference diameter is now part of the buttress and can no longer be measured. The alternate diameter is adopted instead.
Defoliation	The tree has lost all or some of its foliage, leaving apparently dead branches that may arise from insect attack, bats, foliage eating mammals, drought, after fire, etc.
Dead tree	The tree is apparently dead, but remains standing.
Diameter unreliable	The diameter measurement is unreliable because of large climbers, splits, damage or wounds at the point of measurement.
Excrescences	Lumps of callus or wood bulge from the stem, sometimes bearing epicormic branches. Commonly caused by insect or fungal attack.
FB	Fluted bole: The bole has substantial concave sections up its length and cannot be properly measured.
FD	Fire damage: The tree shows evidence of charring of the bark or wood.
FU	Fungi: Fruiting bodies of fungi are growing from the stem or surface roots.
FW	Flowering: This is noted if the tree is in flower, or has recently flowered and is bearing or dropping fruit.
IN	Ingrowth: This code is used only at remeasurement (not initial demarcation). The tree has appeared above the measurement limit and is being marked and measured for the first time.
LD	Logging damage: The tree has suffered damage obviously attributable to logging.
LS	Logged stump: A sawn stump, indicating logging, is found at the tree's coordinates.
LT	Leaning tree: The tree has been pushed or has fallen from its normal position and is some degrees from the vertical.
NT	No tree: The tree is missing. There are no indications of the tree at the required coordinate.
OD	Optical diameter measurement: Used when the trees diameter has been measured by relascope or dendrometer, rather than by tape.
RB	Rotten buttress: The butt or buttress is rotten and partially hollowed.
RT	Rotten timber: Evidence of rot entering the tree from visible rotten pockets left by broken branches.
ST	Strangled tree: The tree has been invaded and its crown dominated by stranglers. Normal diameter measurement will be impossible if this code present.
TF	Tree fallen: The tree has completely fallen.
US	Unknown or uncertain species identification.

Appendix 3: Recording form.

Permanent Sample Plot
Field Data sheet for trees ≥ 10 cm dbh

Ketua Kumpulan:

Ahli Kumpulan:.....

Pencatit:.....

Keterangan Plot

Plot No:	GPS reading		
FMU Name:	A		
Name of Licence:	B		
FMU size:	C		
Coupe:	D		
Size of coupe:	Bearing		
Block:	AB		
Date establishment:	AD		
Assessment: 1 st , 2 nd , 3 rd , 4 th	Elevation:		
Forest type/Age :	A:	C:	
Last harvesting year:	B:	D:	

Ecological description of plot:

1. Presence of skid trail: List of quadrat number effected

.....
.....

2. Occurrences of : permanent stream, seasonal creek.....(please tick of occur)

3. Forest gap due to recent big tree fall: If yes in which quadrat

.....
.....

4. Topographical condition of plot:

Steep slope: degree of steepness in which quadrats:

.....
.....
.....

Undulating:

.....
.....

5. General description of surrounding forest and vegetation:

.....
.....
.....
.....

6. Distance of plot from access road (access point):

7. Others:

No	Tree No	Quadrat No	Coordinate		Plant name (Vernacular name/Species/Family)	DBH	Height		TCC
			X (AB)	Y (AD)			Total	Bole	
1									BT, LT, FT(.....), HB (.....m), TD:.....
2									BT, LT, FT(.....), HB (.....m), TD:.....
3									BT, LT, FT(.....), HB (.....m), TD:.....
									BT, LT, FT(.....), HB (.....m), TD:.....

Footnote: BT: Broken top; LT: Leaning Tree; FT: Forked Tree; TD: Tree deformed at POM

Appendix 4: Recording form for saplings (1 – 9.9 cm dbh).

Permanent Sample Plot Field Data sheet for saplings

[illegible]

3. Tree census in tropical secondary forests

T. Ichie

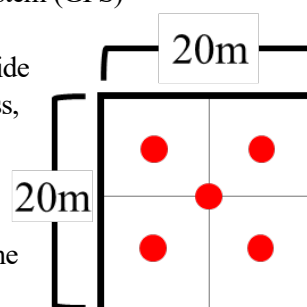
What is a secondary forest?

It is difficult to define a secondary forest precisely. When a forest is in the process of secondary succession after its vegetation has been strongly or frequently disturbed by natural or man-made causes, it is called a secondary forest. Secondary succession is the transition from a previous form of land use, starting with the leftover soil. All forests, except old-growth forests and plantations, can also be defined as secondary forests. Both natural and naturally regenerated forests could be called secondary forests during the period before they became primary forests (climax and old-growth forests).

1. Plot setting

Equipment: 50-, 20-, and 10-m tapes, boundary stakes, a Global Positioning System (GPS) device, a compass, a laser range finder, plastic tape, stationery, and a field notebook.

At least six 20×20 m plots should be prepared in various secondary forests, inside and around each national park, with different soil conditions, forest biomass, biodiversity, fallow duration, and disturbance history. Boundary stakes should be driven into the four corners of each plot, which is surrounded with plastic tape after measurements of 20×20 m are recorded using a compass and a laser range finder (also called compass surveying). The latitudes and longitudes of the four corners of the plot are determined using GPS and recorded in the field notebook.



2. Tree census on the plot

Equipment: Measuring tape (10 m), number tape, a staple gun, labeling tape, a glass fiber rod with a sickle (20 m), a laser range finder, large plastic bags, pruning scissors, stationery, and field notebook. The steps involved in the tree census survey are described as follows:

- a) In the top left corner of the new page of the field notebook, the following information is written before starting the survey:
 - Name of the national park,
 - Name of the surveyor,
 - Weather conditions,
 - Plot number, and
 - Date and time at the start of the survey.
- b) All individual trees on the plot that are more than 15 cm in girth at breast height (GBH, Photo A) are numbered.
- c) The following information regarding each tree should be recorded in the field notebook:
 - Tree number,
 - GBH (1.3 m above ground level, measured on the upper side of the slope), and
 - Scientific, local, or both names of a tree species (when it can be determined in the field).



When measuring the GBH, the measuring tape is strongly pulled several times to ensure no deflection or

kink, and the value is recorded (Photo B). If the trunk leaned, the plane created by the measuring tape around it is ensured to be perpendicular to the central axis of the individual. Assuming that the plane is a circle, divide the circumference by the circumferential ratio to obtain the diameter at breast height.

- d) The height of the largest tree on the plot is measured using a laser rangefinder and recorded in the field notebook.

- e) When the species name of an individual tree is not clear in the field, at least two twigs with several leaves from its branches are collected using a glass fiber rod with a sickle attached to its end, a labeling tape with an ID number is fixed to the collected samples, and all

samples are placed in a large plastic bag. Subsequently, the samples are brought to the research accommodation, and specimens are prepared for identification.



3. Measurement of the light environment

Equipment: digital camera with fisheye lens (or omnidirectional (360°) camera, Photo C), monopod, level, stationery, and the field notebook

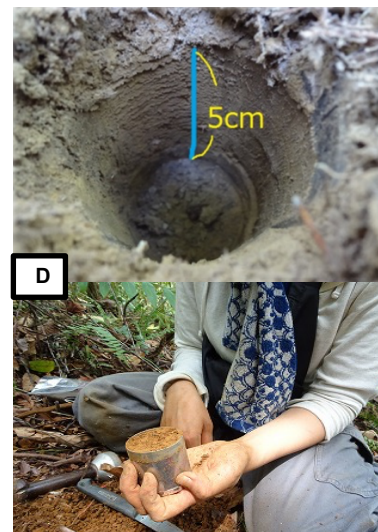
- a) The 20 × 20 m plot should be divided into four 10 × 10 m subplots each.
- b) All-sky images should be captured 1.2 m above ground level at five points: the center point of each sub-plot and that of the 20 × 20 m plot (see the figure on the previous page).
- c) Canopy openness (%) is calculated using specific software, such as CanopOn2 (Takenaka 2009, <http://takenaka-akio.org/etc/canopon2/>). The mean value of the five points should be considered as canopy openness on the plot.



4. Soil survey

Equipment: Soil sampler, scoop, steel tape, paper bags, and plastic zipper bags.

- a) Soil samples are collected from the same five points for light environmental measurements of each plot to determine the soil moisture content, soil properties, and soil nutrient content.
- b) To measure soil moisture content, 100 ml of soil is collected from the 0–5 cm deep surface soil layer using a soil sampler, after removing litter or root mats from the A0 layer, and then placed in a paper bag, recording the plot name and the locations of the five points (Photo D).
- c) Using holes made during surface-layer soil sampling, approximately 100 g of soil is collected with a scoop from each sampling point in the surface layer 0–5 cm deep. Soils from the five points are placed in the same paper bag and mixed for use in soil property and nutrient analyses.



5. Processing of specimens and soil samples in the research accommodation

Equipment: newspapers, plant press (plywood or wire net), straps, pruning scissors, electronic balance, bedding dryer (if available), plastic zipper bags, large plastic bags, stationery, and the field notebook

Plant specimens (Photo E)

- On the same or the following day, the plant specimens are prepared for identification in the research accommodation.
- A folded piece of newspaper should be placed on one of the sheets of plywood, then the paper is opened, and the plant to be pressed is laid out on the paper. The newspaper is closed over the plant, and the sampling date, plot name and number, plant/ tree number, and local name (when known) are noted on the newspaper (additional information is recorded in the field notebook).
- The closed newspaper is covered with another piece to absorb moisture. Subsequently, another newspaper piece is opened, another plant is laid out on this paper. The paper is closed and then covered with another newspaper piece.



This process is repeated for all plant specimens on the same plot, stacked together.

- The second sheet of plywood is placed on top of the stack.
- Straps are used to fasten the stack of plywood and newspaper tightly.
- The pressed specimens are placed in a large plastic bag with a hole at the outlet and dried in a bedding dryer (Photo F).
- Newspaper pieces used for absorbing moisture are changed daily, at least for one week.
- Subsequently, the specimens are dried in a drying oven and used for identification.

Soil sample processing

- Soil moisture content is determined by measuring the fresh weight of soil at the facility. The soil samples are brought to the laboratory and dried for at least 24 h at 105°C to measure their dry weight.
- The soil moisture content is determined by applying the following formula;

$$\text{Soil moisture content (\%)} = (W_r - W_s) / W_r \times 100$$

W_r: Fresh weight, W_s: Dry weight
- The mean value of the five sampling points in a plot is taken as the soil moisture content of the plot.
- The soil samples used for analyzing the properties and nutrient content are air-dried in an accommodation or laboratory and stored for later analysis.

F. Sample drying by bedding dryer



6. Model plan

The following itinerary is suggested for a 12-day field study to be conducted by four individuals: two researchers and two local assistants (or students).

Day 1: Consideration and determination of plot locations

Day 2: Establishment and survey of Plot 1

Day 3: Establishment and survey of Plot 2

Day 4: Establishment and survey of Plot 3

Day 5: Preliminary day

Day 6: Establishment and survey of Plot 4

Day 7: Establishment and survey of Plot 5

Day 8: Establishment and survey of Plot 6

Day 9: Preliminary day

Days 10-12: Plot setting if required by survey in old-growth forests or further survey, depending on the situation

4. Trail census for *Macaranga* trees

U. Shimizu-kaya

1. *Macaranga* — the plant systematics and the relationships with insects

Macaranga Thouars is a Paleotropical genus in the family Euphorbiaceae, with the greatest diversity in Southeast Asia. On Borneo Island, at least 50 species of shrubs or small trees, up to 35 m tall, are distributed in 8 sections/ gestalt groups (Whitmore 2008). Hitherto, 47 species of *Macaranga* have been reported from Sarawak; however, their systematics remain incomplete, particularly for species complexes in mountainous regions. Moreover, the ecological aspects of how closely related species coexist in the same forest habitat are not fully understood.

In contrast, at least 18 species of myrmecophytes belonging to this genus are distributed in Sarawak. Myrmecophytic *Macaranga* trees house arboreal ants, which are called plant ants, inside their hollow stems and usually provide nutrient resources, such as extra floral nectar, for the ants (Shimizu-kaya et al. 2015). Most plant-ant species of *Macaranga* myrmecophytes belong to the genus *Crematogaster* (Feldhaar et al. 2016). The symbiotic relationships between *Macaranga* and *Crematogaster* plant-ants are highly species-specific. In addition to species-specific interactions, defense strategies against herbivorous insects differ even among closely related and sympatrically distributed myrmecophytic *Macaranga* species. Consequently, the community structure of insect herbivores on *Macaranga* myrmecophytes differed among the plant species.

Thus, the census surveys conducted for *Macaranga* at various points in Sarawak would provide data related to plant systematics, phytogeography, the ecology of plant communities, and plant–insect interactions, as well as the evolutionary ecology of *Macaranga*–*Crematogaster* mutualism.

2. Field census

2-1 Tools

Writing materials, field notebook, park maps, zip bags (various sizes), shears (handy and for high branches), 2.0 mL tubes filled with 100% and 70% ethanol, forceps, camera, tea bags, and silica gel.

2-2 Observation along the park trails

Most *Macaranga* species are pioneering. Therefore, park trails providing light conditions in forests are convenient for studying *Macaranga*. For each *Macaranga* tree growing within 2 m width on both sides of the trail, the following data should be recorded while walking along the trails: 1) ID number, 2) position on the trail/distance from stat point, 3) type of habitat, 4) *Macaranga* species, 5) tree height, 6) growth stage, and 7) presence/absence of symbiotic ants. Pictures of the trees are then captured.

2-3 Collection of plants

Dried voucher specimens

For morphological identification, branches with several leaves are collected from each tree. Branches with flowers or fruits are preferred. The plant samples are preserved in zip bags. To prepare dried specimens, please see the previous section: I-3. “*Tree census in tropical secondary forests.*”

Dried leaf fragments for DNA analysis

Leaf fragments are collected from each tree for molecular analysis. For details on collection and preservation, see the the previous section: I-1. “*A standardized belt-transect survey method.*”

2-4 Collection of insects

Symbiotic ants

Symbiotic ant workers are collected from the surface of each myrmecophytic tree using forceps. To observe the morphology of dried specimens, the ants are preserved in 2.0 mL tubes in 70% ethanol. For molecular analysis, the ants are preserved in 100% ethanol. The label for the tree ID, written using a pencil, should be included in each tube.

3. Proposed schedule

The census survey is conducted once for all park trails in each park. Thus, the required time depends on the number of *Macaranga* trees and the number and length of trails. Typically, at least two researchers require three to seven days in a park.

4. References

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5. Useful literature

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II. Fungi

1. Species diversity of polypores

S. Yamashita

Polypores

The polypores produce conspicuous fruiting bodies that are readily visible to the naked eye. Typically, these fungi form tough and persistent fruiting bodies characterized by poroid or lamellate hymenophores, which emerge from substrates, such as fallen trees, twigs, stumps, and occasionally from the forest floor. The morphology of the polypore fruiting bodies is highly variable. Certain species exhibit resupinate forms that appear as thin layers adhering to the undersides of woody debris, whereas others develop ungulate structures that protrude laterally from stumps or fallen trunks. Polypores are polyphyletic groups that include Hymenochaetales, Polyporales, and Russulales. Most polypore species are saprotrophic. The fruiting bodies of polypores collected in Sarawak from 1954 to 2003, which are stored in the fungarium of RDD and FDS, are listed with pictures (Yamashita et al. 2018). The methods used to assess the polypore fungal diversity are outlined below.

1. Setting transects or plots

Yamashita et al. (2015) reported that the species richness of wood-decaying polypores in the tropical rainforests of Malaysia is likely to be considerably underestimated unless field surveys are conducted on at least three separate occasions within a plot exceeding 1.5 hectares in area. When the sampling effort, defined as the product of the survey area and the number of sampling occasions, is constrained, prioritizing a larger survey area with fewer sampling events is a reasonable compromise to maximize species coverage. Based on the findings of this study, establishing multiple fixed sampling units, such as line transects, square plots, and circular plots, within a target forest stand is recommended. The cumulative area of these sampling units should exceed 1.5 hectares to ensure adequate representation of polypore diversity. Measuring tapes, stakes, or markers, a GPS receiver, a field notebook, and writing implements are needed.

2. Collection of fungal fruiting bodies

Before starting the collection of fungal fruiting bodies, the following information should be recorded on the left-hand page of a new spread in the field notebook: the name of the national park, names of the collectors, weather conditions, and identifier of the sampling unit.

All fruiting bodies of polypores observed within the boundaries of the designated line transects or plots should be collected. In cases where multiple fruiting bodies of the same fungal species are present on a single substrate, a maximum of five individual fruiting bodies should be collected to represent each species (Fig. 1). For each specimen, the pre-assigned specimen number (written on a paper bag before the start of the fieldwork), substrate type, such as soil, fallen trunks, twigs, stumps, standing dead trees, or living trees, and the diameter and decay class of the substrate from which the fruiting body emerged must be recorded. The decay classes should be categorized into five levels:

- Class 1 – Solid coarse woody debris (CWD) with intact bark;
- Class 2 – Solid CWD with the bark beginning to detach;
- Class 3 – Non-solid but firm CWD;



Fig. 1 Large numbers of the fruiting bodies of *Microporus vernicipes* emerging from a fallen trunk.

Class 4 – Soft and rotten CWD into which a nail can be easily inserted; and

Class 5 – Soft and rotten CWD that collapses easily.

Each sample should be placed in a paper bag (Fig. 2) and transported to the laboratory for further examination. Paper bags are highly recommended for keeping fungal fruiting bodies with high moisture content, as they tend to deteriorate rapidly when kept in plastic (vinyl) bags owing to poor ventilation.



Fig. 2 Paper bags

3. Laboratory work to be performed immediately after field sampling

Considering the importance of DNA data for accurate taxonomic identification and diversity estimation, tissue samples for DNA extraction should be prepared from each collected specimen. To prevent the deterioration of fungal fruiting bodies, laboratory procedures for DNA sampling should be initiated as soon as possible, ideally within 24 h of field collection.

Before commencing the laboratory work, the workspace should be disinfected by spraying 70% ethanol onto the surface and wiping it with a clean paper or cloth soaked in the same solution. Forceps and knives must be sterilized by wiping with ethanol-soaked paper and subsequently flaming.

Once sterilized, the fruiting body should be divided into two or more sections, and the fungal tissue should be excised from the context using sterilized instruments. Extracted tissues should be placed in tubes containing 99.5% ethanol. Each tube is labelled with the corresponding specimen number and stored in a freezer until further processing.

After the tissue sampling for DNA extraction, the remaining fruiting bodies should be placed in paper bags. The specimen number, date of collection, name(s) of the collector(s), and study site should be clearly indicated in each bag. The fruiting bodies should then be dried at 60°C for a minimum of 72 h. After drying, the specimens are to be deposited in a fungarium. A spray bottle containing 70% ethanol, forceps (Fig. 3), a knife, a tube stand, clean paper (KimWipes), 2.0 mL tubes containing 99.5% ethanol, and a cigarette lighter or gas burner are needed.



Fig. 3 Forceps used in tissue sampling in the laboratory. The lower forceps are used for tearing apart small pieces of context from fruiting bodies. The upper one is used for handling small pieces of fruiting bodies during laboratory procedures.

4. Morphological observation

To achieve the species-level identification of polypore fungi, dried specimens must be carefully examined. The thickness of the context and tubes or lamellae should be measured using a ruler, and the number of pores or lamellae per millimeter should be counted under a stereomicroscope.

For the microscopic observation of spores and hyphal structures, at least three sections should be prepared and treated as follows:

1. Potassium Hydroxide (KOH) Treatment:

One section is placed onto a glass slide, and a drop of 3–5% (w/v) KOH is applied adjacent to this section. A cover glass is gently placed to avoid any air bubbles. The cover glass is gently rubbed with an eraser to loosen the hyphae. The colors of the hyphae and spores are observed and recorded. The sizes of spores, setae, and setal hyphae are also documented.

2. Phloxine Staining:

The second section is stained with 1% (w/v) phloxine. Under this treatment, the generated hyphae typically

appeared pink. The presence or absence of clamp connections in the generative hyphae should be recorded.

3. Melzer's Reagent Test:

The third section is used to assess their reactions to Melzer's reagent. Reactions are categorized as amyloid, dextrinoid (pseudo-amyloid), or inamyloid (no reaction). Reaction types should be recorded for both spores and hyphae.

For further methodological details, please refer to Ryvarden & Johansen (1980), and other relevant studies.

5. Storing samples

Fungal fruiting bodies dried at 60°C for a minimum of 72 h should be stored in an air-conditioned room. Therefore, insecticides and fungicides should be periodically administered. Samples intended for molecular analyses, including DNA extracts and the tissues used for DNA extraction, must be stored in a freezer at –20°C.

6. Model plan

Assuming that two researchers conduct a field survey over 12 d to elucidate the species diversity of polypores in a national park, the model plan is shown below:

Day 1: field investigation for setting fixed sampling units;
 Day 2–9: field sampling and lab work; and
 Day 10–12: walking along trails outside the fixed sampling units and collecting polypore fungal fruiting bodies that have not been recorded from inside these units.

References

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2. Phylogeographic study of *Ganoderma* and *Amauroderma*

S. Yamashita

Ganoderma and *Amauroderma*

Ganoderma and *Amauroderma* are formerly classified within the family Ganodermataceae; however, recent taxonomic revisions placed them within the family Polyporaceae. These genera are closely related to *Polyporus*. They form large, conspicuous fruiting bodies on fallen trunks, stumps, dead standing trees, living trees, and the ground.

Collection of fungal fruiting bodies

Before the collection of fungal fruiting bodies, the following information should be recorded on the left-hand page of a new spread in the field notebook: name of the national park, date, names of the collectors, prevailing weather conditions, and name of the trail.

The fruiting bodies of *Ganoderma* and *Amauroderma* should be surveyed by walking along the designated trails in national parks. The latitudes and longitudes of both the start and end points of each trial should be recorded using a global GPS receiver. All the fruiting bodies of these genera observed during the trials should be collected. For each specimen, the following data must be recorded: specimen number (written on a paper bag before the start of the fieldwork), substrate type, such as soil, fallen trunks, twigs, stumps, standing dead trees, or living trees, the diameter and decay class of the substrate, and the GPS coordinates of the collection site. Each sample is placed in a paper bag and transported back to the laboratory. When multiple fruiting bodies of the same fungal species are present on a single substrate, a maximum of five individual fruiting bodies should be collected per species from that substrate.

For information on laboratory works and the following procedure, please refer to the text presented in Sections II-1-3 to II-1-6 and the references therein.

3. Procedure for collecting and sorting ectomycorrhizal samples

H. Sato

Ectomycorrhiza

Mycorrhizae are symbiotic associations established between the roots of most terrestrial plants and soil fungi. Mycorrhiza is commonly divided into seven types according to the morphology of the root structures formed and the organisms involved (arbuscular mycorrhiza, ectomycorrhiza, ectendomycorrhiza, ericoid mycorrhiza, orchid mycorrhiza, arbutoid mycorrhiza, and monotropoid mycorrhiza). Among them, ectomycorrhizae are characterized by the formation of fungal hyphae sheaths surrounding the root (mantle), the growth of hyphae between root cortical cells (Hartig net), and the growth of extramatrical mycelia. Most ectomycorrhizal fungi belong to the Dikarya subkingdom (Ascomycota and Basidiomycota), and the host plants are tree species that are dominant in temperate and tropical forests, such as Pinaceae, Fagaceae, Betulaceae, Dipterocarpaceae, and Myrtaceae.

Collection of ectomycorrhizal samples

Instruments: Trowel, plastic bag, carrier bag (cool bag), GPS, writing tools, and a notebook.

- Using a trowel, a soil core (10 × 10 × 10 cm) is collected from the fermentation and humus layers at each sampling point. To efficiently collect ectomycorrhizal samples, samples should be collected within a radius of 3 m of the ectomycorrhizal tree. The collected soil cores are placed in plastic bags on which the sample name and GPS ID are written. The plastic bags are then placed in a carrying bag. If not returning to the laboratory immediately, they should be placed in a cool bag. At each sampling point, location information is recorded using the GPS device.
- The required number of sampling points varies according to the purpose of the study; however, 20–50 sampling points are often sufficient. A distance of at least 3 m is needed between any two sampling points to avoid duplicate sampling.
- Subsequently, the samples are stored in a freezer until further processing. If moving on to the next step by the end of the next day, the samples can be stored in a refrigerator.

Washing and sorting of ectomycorrhizal root tips

Instruments: Stainless-steel sieve (2.0-mm mesh), Tweezers, Stereo microscope, vortex mixer, 70% ethanol, Aerosol OT, sterilized water, cetyltrimethylammonium bromide (CTAB) buffer, and Kim towels.

- If the soil cores are stored in a freezer, they should be stored overnight in a refrigerator to defrost the soil.
- Terminal roots (>1 cm) are collected from the soil, placed in a stainless-steel sieve, and washed with tap water. The required number of terminal roots changes according to the purpose; however, 20 roots are often sufficient.
- Using tweezers under a stereo microscope, almost all ectomycorrhizal root tips (1–2 mm) are collected from each terminal root and their images captured. Ectomycorrhizal root tips can be distinguished based on the mantle and extramatrical mycelia.
- All root tips are collected from the same terminal root into a 1.5 mL tube using tweezers. To identify the ectomycorrhizal fungi of each root tip, place individual root tips in different tubes (not recommended because the number of samples will be enormous).
- After adding ca. 500 µL of 70% ethanol in each 1.5 mL tube, vortex tubes for 30 s at maximum speed. Root tips are placed on a Kim towel using tweezers to remove water.



Photos. Representative examples of ectomycorrhizal root tips.

- Using tweezers, the root tips are placed in fresh 1.5 mL tubes containing approximately 500 μ L of 0.005% Aerosol OT solution each, and the tubes are vortexed for 30 s at the maximum speed. Subsequently, the root tips are placed on a Kim towel to remove adhering traces of the Aerosol OT solution.
- Next, the root tips are transferred to fresh 1.5 mL tubes containing approximately 500 μ L of sterilized water, and the tubes are vortexed for 30 s at the maximum speed. Subsequently, the root tips are wiped dry by placing them on a Kim towel.
- Using tweezers, the root tips are again transferred to fresh 1.5 mL tubes containing approximately 100 μ L of CTAB buffer. These tubes can be used for DNA extraction. When DNA extraction is not performed immediately, the tubes are stored in a freezer.

Notes:

- As it is difficult to perform the washing and sorting processes outside the lab, it is necessary to return to the lab within 1–2 days after collecting the soil cores.
- It often takes several hours to complete a field survey at one survey site.
- Ectomycorrhizal root tips can be sorted accurately if the root samples are observed under an optical microscope. To do this, a microscopic section of the root tips was made using a razor and dry elder pith and confirmed the presence of a mantle and Hartig net. However, observation under an optical microscope often requires a fair amount of time, and it is impractical to perform this for all root samples. Thus, an optical microscope should be used only for partial samples (it does not matter if you sort ectomycorrhizal root tips under a stereomicroscope).

4. Epifoliar fungi (Meliolales)

Y. Ando

The order Meliolales belongs to the class Sordariomycetes in the fungal phylum Ascomycota. This order comprises two families: Armatellaceae and Meliolaceae. These are known as plant pathogens that occur on the surface of leaves, stems, branches, or petioles of vascular plants and are called “black mildews” or “dark mildews.” The species included in the order Meliolales are believed to possess host specificity; therefore, it is necessary to identify their host to identify the fungal species.

Installation of survey plots

- As Meliolales fungi are considered to have a high host specificity, species diversity is thought to depend on plant diversity. Therefore, field surveys of biodiversity for Meliolales fungi should be conducted using the vegetation survey method.
- Tools used: tape measure (50 m), stake, GPS receiver, writing equipment, and field notebooks.
- At the representative vegetation in a national park, 20 transects of 5 m × 100 m should be prepared (total area: 1 ha), and plots should be provided for each transect of 10 m length.
- GPS information will be acquired at both ends of each transect.
- Numbers are assigned to each transect and plot to distinguish them.

Collection of samples

- Samples should be collected from heights within reach (using high-twig scissors if necessary).
- Tools to use: loupe, paper sack, writing equipment, GPS receiver, field notebooks, pruning shears, and camera.
- Start collecting after writing down the following items on the upper left of the new page of the field notebook.
 - Draft of survey
 - Name of the national park
 - Investigator's name
 - Number of transects and plots
- Sampling is performed for each plot, and leaves infected with Meliolales fungi (Fig. 1) are collected. Samples are collected separately from each plant, and about 5 to 20 samples (diseased leaves) are collected from each plant.
- If the host plant cannot be identified at the site, plant organs, such as branches and flowers, are collected (or take photographs of them) for later identification.
- Each time a sample is collected, the following items should be written in the field notebook:
 - Sample number
 - GPS number
 - Name of host plant
- The collected samples are placed in a paper bag, the sample number is written, and the samples are transported to the laboratory.
- If several species occurred on the same host plant, each type of diseased leaf is collected and placed in the same paper bag.
- The survey should be conducted in 6 min per person per plot.



Fig. 1 Meliolales fungi on various plants.

Sample processing at the accommodation during the survey period (preparation of specimens)

- Tools to use: old newspapers, field press, rope, cameras, and rulers.
- The diseased leaves should be photographed alongside a ruler.
- The collected sample is held in an old newspaper, and the specimen is prepared. The newspapers are changed daily until the sample is dry. When the sample is dry, it is stored in a location with low humidity. It should be noted that there is a high risk of saprophyte contamination in samples with high water content, such as samples collected after rain or thick leaf samples.
- Meliolales fungi may also be infected with hyperparasitic fungi. Therefore, pick up of myceliums or ascocarps of Meliolales fungi for DNA extraction is performed under a stereomicroscope after the samples are brought back to the laboratory.

Model plan

The work plan for a 12-day field survey by two people (referring to the method of vegetation survey) is as follows:

-
- Day 1 – Decision on where to install transects;
 - Day 2 – Installation and investigation of three transects;
 - Day 3 – Installation and investigation of three transects;
 - Day 4 – Installation and investigation of four transects;

- Day 5 – An optional extra day preliminary date;
 Day 6 – Installation and investigation of three transects;
 Day 7 – Installation and investigation of three transects;
 Day 8 – Installation and investigation of four transects;
 Day 9 – An optional extra day preliminary date; and
 Day 10–12 – Supplemental survey. The trail outside transects should be examined, and diseased leaves should be collected.
-

Sample processing in the laboratory

Morphological observations

- The dried specimen prepared in the above process is observed.
- Tools to use: slide glass, cover glass, mounting fluid, tweezers, scalpel, razor, and burner.
- Observation under a stereomicroscope
 - The sign should be photographed.
 - The appearance of colonies on the leaves and the presence/ absence of ascomata and setae are observed.
- Observation under a light microscope
 - A mounting fluid, such as water or shear's solution, is dropped onto a slide glass, and the fungal structures, including ascomata, hyphopodium, and setae, are suspended in the mounting fluid. Subsequently, the specimens are mounted under a cover glass to observe them under a microscope.
 - Ascomata, sections of ascomata, asci, ascospores, setae, hyphopodium, and phialide are imaged, and their morphology and size are recorded (see Fig. 2).

Molecular phylogeny

DNA extraction

- The dried specimen prepared in the above process is observed.
- The samples should be observed under a stereomicroscope and look for fresh colonies that are free of hyperparasitic fungi and other saprophytic fungi. When a colony is found to be in good condition, it is picked up with flame-sterilized tweezers or a scalpel and suspended in a DNA extraction reagent in a 1.5 mL tube. The suspended fungal structures are ground using a pestle, and DNA is extracted according to the manufacturer's protocol.

Store the samples

- Dried specimens: Store in a low-humidity environment at a temperature of 20°C or less.
- Extracted DNA samples: Store in a freezer at –20°C.

Useful literature

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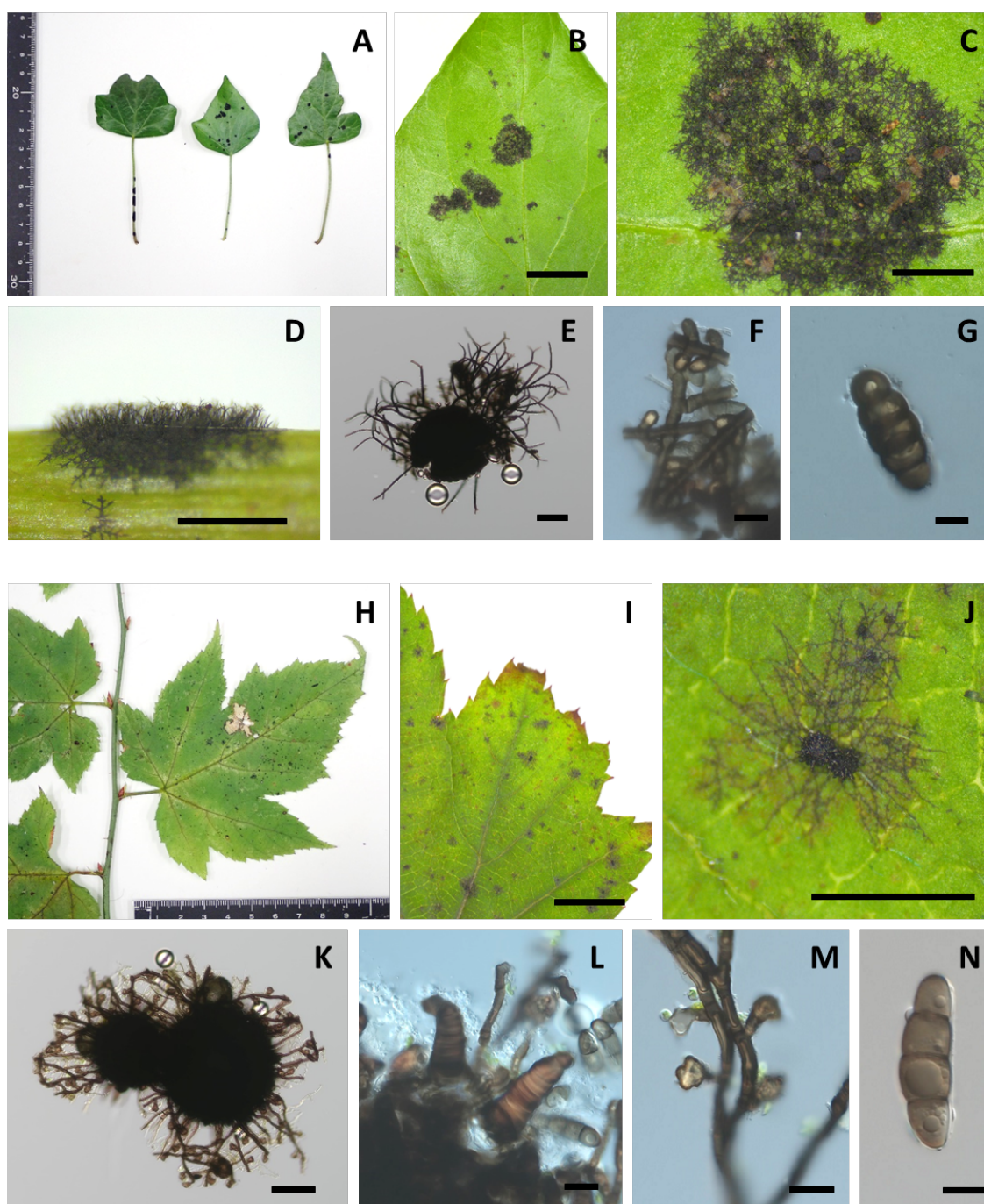


Fig. 2 Morphological characters of Meliolales fungi. A, B, H and I: disease symptoms (bar = 5 mm). C and J: colony (bar = 1 mm). D: hyphal setae (bar = 1 mm). E and K: ascomata (bar = 100 μ m). F and M: hyphopodia (bar = 20 μ m). G and N: ascospore (bar = 10 μ m). L: appendage on ascomata (bar = 20 μ m).

III. Insects

1. General methods for quantitative collection for insects

H. Kawagoe and T. Itioka

1. Light-trapping

Several species of nocturnally flying insects can be efficiently collected by light trapping, a method of insect collection that uses ultraviolet (UV) light. While various types of UV light are available for entomological trapping, considerable attention should be directed to potential differences in collection efficiency. Specifically, quantitative comparisons of temporal and spatial population fluctuations require the standardization of light emission wavelengths, meteorological conditions (including temperature, humidity, wind speed, and rainfall), lunar phase, and microenvironmental conditions of trap placement. To minimize the influence of temporal environmental variability, such as meteorological conditions, it is necessary to conduct repeated sampling over a short period (e.g., several days). Furthermore, to reduce microenvironmental bias, a sufficient number of sites must be established to ensure robust spatial replication. When setting up multiple traps simultaneously, prepare a device that utilizes a transparent panel to intercept insects attracted to the light source, directing them into a bucket or other container filled with an insecticidal chemical. The bucket should be filled with an appropriate amount of ethanol or propylene glycol, or with newspaper or tissue paper moistened with ethyl acetate, to weaken the insects and prevent them from escaping (Fig. 1).

If the primary objective of light trapping is to conduct a taxonomic inventory rather than a quantitative comparison, the requirements for temporal or spatial replication may be relaxed.

Light traps like the one illustrated in Fig. 1, which trap insects by placing insecticidal chemicals in containers, are not suitable for collecting moths. Contact with these chemicals often disrupts the scale arrangement on the wings of moths. This degradation obscures the color patterns necessary for accurate species-level identification. For the effective collection of moths, it is recommended to hang a white sheet next to the light source and manually capture the specimens upon arrival (Fig. 2) using an insect net or insect-killing bottle.

To maximize capture yields, light trapping should be scheduled on days with low lunar illumination (e.g., full moon) and should be performed under low-wind conditions. Such considerations are fundamental aspects of forest ecology.



Fig. 1 Light trap set up at the canopy plot in Lambir Hills National Park. This trap is equipped with a rain shelter roof, a UV fluorescent lamp, four transparent acrylic plates for flying insects to collide and fall, a metal funnel, and a plastic container with chemicals to kill and preserve insects. This type of light trap was used in studies such as Kato et al. (1995, 2000), Itioka et al. (2001) and Kishimoto-Yamada et al. (2009). (Photo by Tamiji Inoue)

2. Flight interception trap

Flying insects, mainly Coleoptera, are collected using various types of flight interception traps (FIT). An FIT causes flying insects to collide with a transparent plate, subsequently fall and be captured in a pan or container



Fig. 2 Light-trapping with a white sheet. To capture moths, it is recommended to hang a white sheet and manually capture moths upon arrival using an insect net and killing bottle.

filled with liquid (e.g., propylene glycol or detergent solution). Similar to the light-trap method, FIT also requires considering for biases in the amount and species composition of obtained samples, owing to the specific microenvironment of the location where the trap is set up. However, compared with light traps, FITs can be constructed in large numbers using relatively inexpensive materials, facilitating the simultaneous deployment of large trap numbers within a study site. Furthermore, because FITs operate over several days, regardless of the time of day, it is not necessary to account for minor daily variations in meteorological conditions or interspecific differences in phototaxis and diurnal flight behavior. Consequently, this method is highly suitable for quantitative comparisons of diversity and community structure.

Here, we briefly introduce the FIT sampling method developed by Maruyama (2003) for capturing insects flying near the ground surface (Fig. 7d).

2-1. Materials (*quantity per FIT*) (Fig. 3a, b)

- A3-size transparent plastic document folder (2)
- 30 cm-long bamboo skewers (1–2)
- 70 cm-long orchid wire (bendable metal support for gardening) (2)
- 10 cm-long wire or cable tie (6–10)
- Plastic tray (20 × 15 × 5 cm) (3)
- Propylene glycol (500–700 ml)

2-2. Required tools for trap assembly

- Stapler
- Paper hole puncher
- Cutter knife
- Pliers

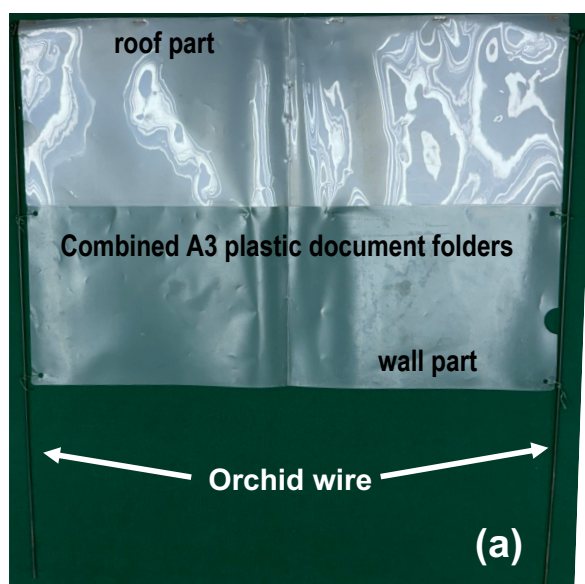


Fig. 3 Materials for assembling a FIT: (a) two sheets of A3-size transparent plastic document folder and two 70 cm-long orchid wires (already combined to form the main body of FIT), and (b) plastic trays and a 30 cm-long bamboo skewer.

2-3. Required materials specimen collection

Paper tea filter bag (Fig. 4)

50 ml tube or bottle

2-4. Assembly and installation of a FIT

(For detailed information, refer to the illustration guide in Maruyama 2003)

- 1) Using a cutter knife, open both clear holders by peeling apart the adhesive joint.
One is used to form the roof, and the other is used to form the wall (Fig. 3a), which serves as the plate that flying insects collide with perpendicular to the ground (Fig. 5).
- 2) Staple the wall sheet to the folded section at the center of the roof sheet (Fig. 5).
- 3) To ensure the wall sheet remains taut and perpendicular to the ground, it must be attached to two posts (70 cm long orchid wires) placed in the ground (Fig. 5). To connect the wall to the two supports, use a hole puncher to make three or four holes (**X holes**) at appropriate intervals on both short edges of the wall sheet (Fig. 6b, c). For a similar purpose, punch holes (**Y holes**) near both ends of the roof sheet where it intersects the wall sheet (Fig. 6a, d).
- 4) Use pliers to bend the ends of the post wires to create loops of approximately 1 cm in diameter. These loops will serve to hang the entire structure (main trap body) (Fig. 7).



Fig. 4 Paper tea filter bags used for collecting insects captured by FITs.

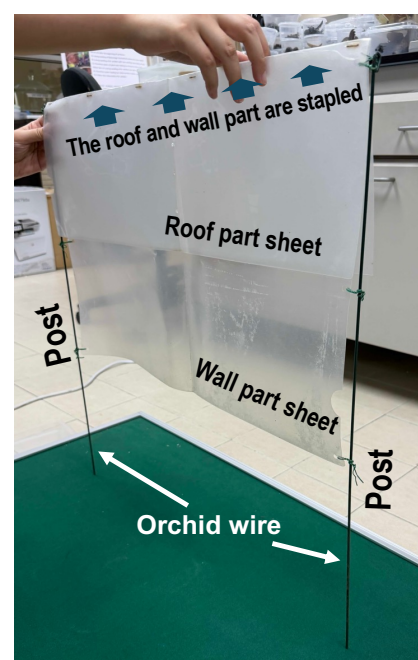


Fig. 5 Main body of the FIT, consisting of the roof sheet, the wall sheet, and the two posts made of orchid wires. The upper part of the wall sheet is sandwiched inside by the roof sheet. The wall sheet is stapled with the roof sheet at the top (along the centerline of the sheet).

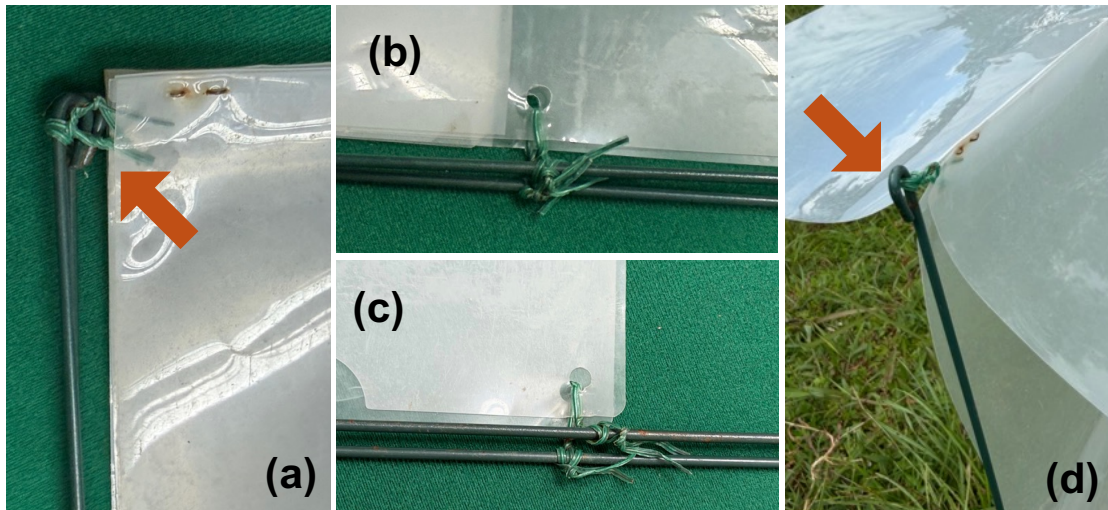


Fig. 6 The joint parts between the post (orchid wire) and the wall sheet. At each part, **X holes** (b, c) and **Y holes** (a) are made and the post and the sheet are tied by wire (or cable tie). The combined sheets (roof and walls) are hung from the loop at the end of the post by a wire (or cable tie) connecting **Y holes** to the loop (a, d) (The arrow indicates the loop).

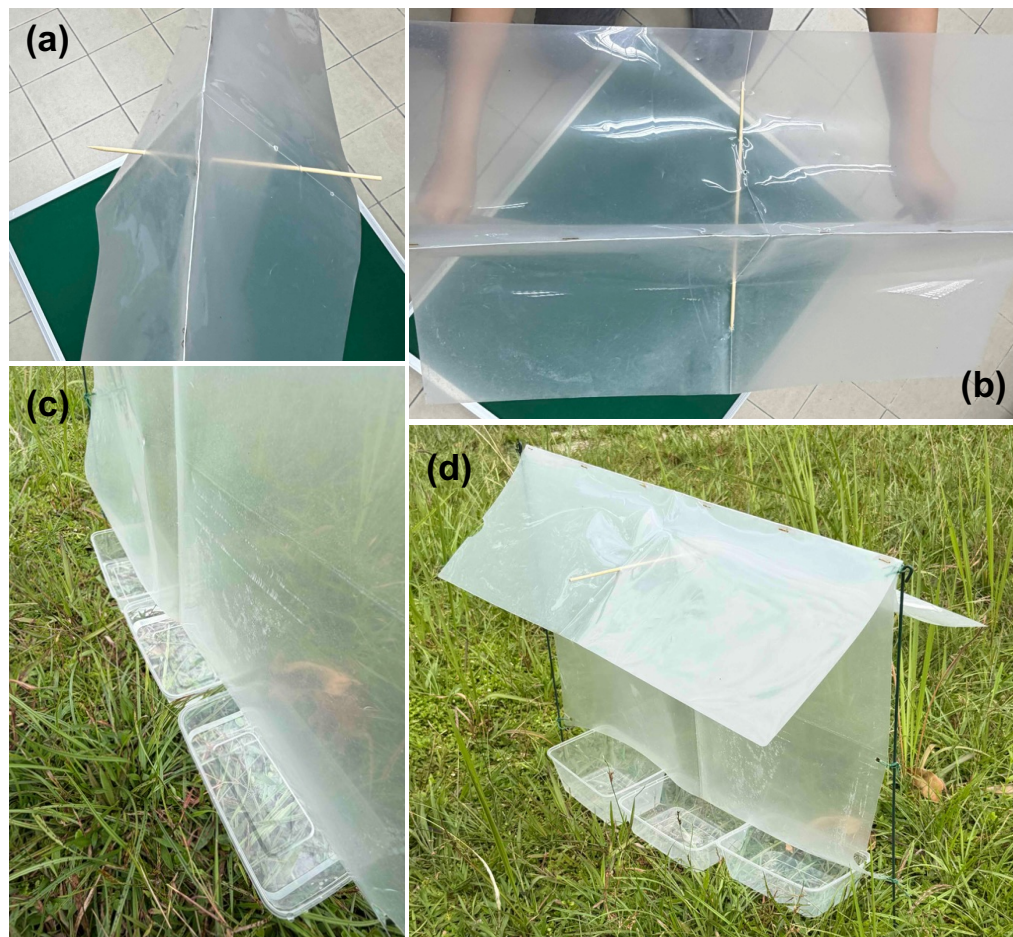


Fig. 7 Adjusting the shape of the FIT for the roof part with a bamboo skewer (a, b) and the capture containers at the bottom (c), and the completed shape (d).

- 5) Use wire (or cable tie) to connect the two holes (**Y holes**) on the roof to the loops at the ends of the two posts. Then, use wire (or cable tie) to connect the 3–4 **X holes** in the wall sheet to the posts so that the wall sheet stays flat.
- 6) Select a level spot on the ground as the trapping site. The positions of the two posts should be adjusted such that the wall sheet stands vertically on the ground while maintaining a flat surface and insert the posts into the ground to secure the entire body.
- 7) Insert bamboo skewers through three points: 1) near the center of the half of the roof sheet separated by the joint section to the wall sheet, 2) near the top of the wall sheet, and 3) near the center of the opposite half of the roof sheet to stabilize the shape of the roof and prevent rainwater from entering the container installed below (Fig. 7a, b).
- 8) Place three plastic trays horizontally below the wall sheet (Fig. 7d).
- 9) Pour propylene glycol into each tray until it is at least 2 cm deep.
- 10) Leave the FIT for 2–7 days.

2-5. Collection of captured insects

- 1) Pour the liquid from the tray along with the trapped insects into a tea filter bag. Any liquid that passed through the tea filter bags should be discarded.
- 2) Transfer the tea bag and any insects captured into an airtight container (e.g., 50 ml plastic tube).
- 3) Insert a data label describing the collection date, location, ID of FIT, and other details into the container.

3. Malaise trap

In addition to FIT, collection methods using Malaise traps (MT) (Fig. 8) also target flying insects. The MT employs a suspended net to interrupt the flight of an insect. By utilizing the tendency of most insects to move upward or towards a stronger light environment when they resume flight or walk after an interruption, the trap is designed to lure insects towards a collection container mounted above the trap (Fig. 8). Compared to FIT, MT can efficiently collect flying insects that do not fall even when their flight is interrupted by a transparent barrier, as well as those that might otherwise avoid such a board. Therefore, while FIT is suitable for collecting coleopteran insects, MT is more suitable for collecting hymenopteran insects.



Fig. 8 A Malaise trap set up in an acacia planted forest.

As with the two trap types, when using this method, it is necessary account for biases caused by the microenvironment of the trap location.

4. Other methods for insect collection

Various methods have been developed for quantitative insect sampling. Quantitative **sampling of soil or litter** is often conducted in combination with various types of extraction devices, such as the **Winkler bag** and **Tullgren Apparatus**, for sampling soil arthropods, including ants. **Pit-fall traps** are used to sample ants and ground-dwelling insects. However, this method may be less effective for sampling ground-dwelling insects in tropical areas compared to temperate regions. This discrepancy is likely due to the substantial lower abundance of these insects in tropical rainforests probably resulting from the lack of humus in the soil. Other methods, such as using various types of **bait trap**, **beating-net**, and **net-sweeping**, could be suitable for sampling particular

insect taxa. Please refer to Nakashizuka & Stork (2002) for more detailed information on other insect collection methods.

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2. Transect census for termites

Y. Takematsu

1. Termites

Termites are eusocial insects belonging to the order Blattaria. They are widely distributed and play a key role in decomposition processes in tropical ecosystems. They are also useful for ecosystem monitoring, particularly because of their functional importance.

2. Standardized sampling for termites

Comparison of biodiversity among several sites requires a standardized sampling protocol and rapid assessment of local termite assemblages that can produce an accurate picture of species composition.

2-1. Preparation and equipment

- Group equipment (Fig. 1):
100-m measuring tape, GPS device, tape or string
- Personal equipment (Fig. 2):
Knife or hatchet, white tray, work gloves, forceps, plastic or glass tubes filled with 80% ethanol, marker, plastic bag



Fig. 1 Group equipment.
100m-measuring tape, GPS device, tape or string.



Fig. 2 Personal equipment.
Knife or hatchet, white tray, work gloves, forceps, tube filled with 80% ethanol (plastic or glass), marker, plastic bag.

2-2. Methods

1) Select the transect site

At each location, select a transect site. The site should be more than 100 m long and 2 m wide and more than 50 m away from open areas (such as houses and roads).

(2) Set the transect

The transect is 100 m long and 2 m wide, and is divided into 40 contiguous sections (each 5×1 m), which are numbered sequentially (Fig. 3).

(2)-1 Decide the starting point and fix the measuring tape with tape or string at the starting point.

(2)-2 From the starting point, one person extends the 100 m measuring tape straight ahead to the end point. At the end point, fix the measuring tape with tape or string.

(2)-3 Record the location information of the starting and end points using a GPS device.

(3) Termite sampling

Each small section is sampled by a trained individual for 30 min. To standardize sampling effort, collectors must work steadily and continuously during each 30-min collecting period. In each section, the collectors search the following microhabitats, which are common sites for termites: 12 samples of surface soil (each about 12×12 cm, to a depth of 10 cm); accumulations of litter and humus at the base of trees and between buttress roots; the inside of dead logs, tree stumps, branches, and twigs; the soil within and beneath very rotten logs; all subterranean nests, mounds, carton sheeting, and runways on vegetation; and arboreal nests up to a height of 2 m above ground level.

“Trained individual” refers to a person who can distinguish and collect termites.

(4) Record sheet and samples

The team leader fills out the record sheet (attached spreadsheet) for each transect.

Date, locality, GPS information for the starting and end points, collectors’ names, and number of collected bottles for each small section are recorded.

All samples from one transect are placed together in a plastic bag.

2-3. *Sample treatment*

• On-site

Place each colony in a separate tube. Immediately after collection, write the section number (1A, 1B, 2A, 2B, ..., 20A, 20B) on the top of each tube.

Put all samples from one transect together in a plastic bag.

• Laboratory

Each tube is opened into a Petri dish, and the termites are separated from debris and soil.

Termites are placed in a glass vial filled with 80% ethanol.

In each vial, place a piece of paper with the location, date, collector’s name, transect number, and section number written in pencil.

After identification, place an identification label written in pencil.

2-4. *Model plan for sampling*

(Assuming a 14-day itinerary for four people)

If one transect survey is conducted over 2 days:

First day	AM: Select site and set the transect; 30 m survey
	PM: 40 m survey
Second day	AM: 30 m survey; finishing
	PM: Random sampling around the transect site

Day 1	Preparation
Day 2–3	One transect + random sampling
Day 4–5	One transect + random sampling
Day 6–7	One transect + random sampling
Day 8	Extra day
Day 9–10	One transect + random sampling
Day 11–12	One transect + random sampling
Day 13	Extra day
Day 14	Finishing

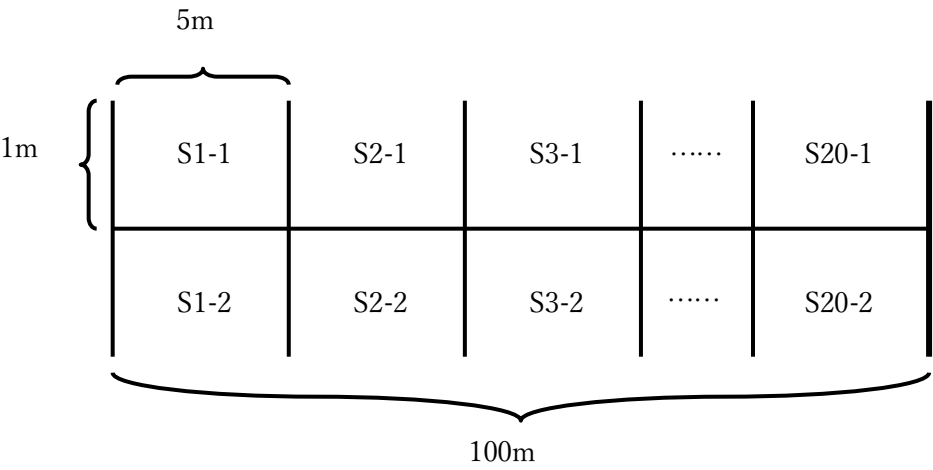


Fig. 3 Transect design.

3. Wild bee survey

U. Shimizu-kaya

1. Survey of bee fauna

Insect–plant interactions are one of the important aspects of forest ecology. In particular, interactions between flowers and flower-visiting insects, including pollinators, in lowland dipterocarp forests have been studied for several decades. However, inventory work and the biogeographical studies of flower-visiting insects in Sarawak have been insufficient. Wild bees are generally major flower visitors in non-agricultural ecosystems, and this is also true in dipterocarp forest in Malaysia (Appanah 1990, Inoue et al. 1990, Nagamitsu & Inoue 1994). Recently, the global declines in bee populations have been reported. Therefore, inventory work for bees in Sarawak should be conducted before it is too late.

For wild bees, quantitative surveys are usually difficult because the number of collected individuals can vary depending on environmental conditions, such as weather, the abundance and species composition of flowers at the time of sampling, and site characteristics. Additionally, the seasonality of the bees in Sarawak remains unclear. Therefore, we focused on conducting as many qualitative surveys as possible in the national parks to show how many species, and what species, inhabit Sarawak.

2. Field survey

2-1. Tools

Writing materials, field notebook, sweeping net, insecticide bottle (50-ml tube or bottle with cut cotton soaked in 5 ml ethyl-acetate), forceps, empty containers (50-ml tubes or Petri dishes), camera, zip bags, shears.

2-2. Sampling points

In addition to wildflowers, gardening and ornamental flowers should also be targeted. Flowering plants are usually found at forest edges, in gaps, and along rivers. On the first day, researchers should walk around the area and select targeted plants for the census. The area surrounding the headquarters of each park may have some flowering plants and can serve as the first sampling point. Additional sampling points should be added depending on the presence of flowering plants.

2-3. Collection

Fine and calm weather, between 9:00 to 15:00, is ideal for sampling. Upon observing bees attracted to flowers in bloom, collect as many specimens as possible using a sweeping net (Fig. 1). Captured individuals are killed in the insecticide bottle and preserved separately in empty containers according to the plant species they visited. Random sweeping of a flower should be restricted at the end of sampling on that flower, or when no bees are visible, as it may damage the plant. Targeted plants are photographed, and samples are collected in zip bags for species identification.

2-4. Recording

Taxonomic identification of the targeted plants may be difficult in the field. Therefore, each plant species should ideally be assigned a serial number. The serial number should be written down on the containers for collected bees and on zip bags for collected plant samples. Sampling points, date, time, weather, and the number of targeted plants should be also recorded.

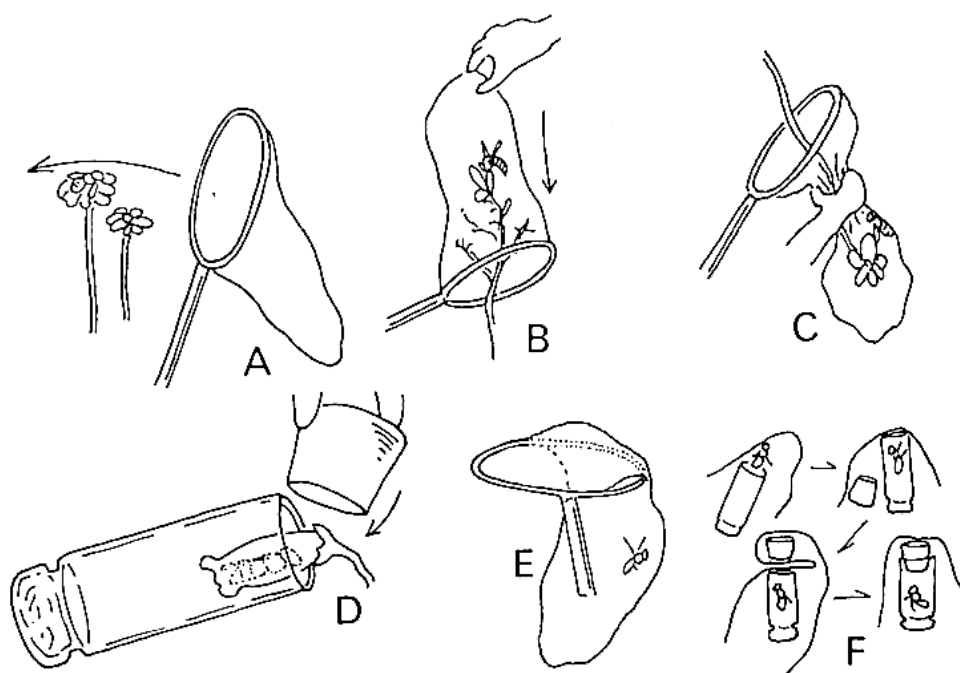


Fig. 1 Illustrations of net sampling (based on Sakagami et al., 1974). A: Normally sweep from the side of the flowers. B: For shrubs, thorny, ornamental, cultivated, or rare plants, gently cover the net over the bees and flowers to make the bees fly into the net. C: For hanging flowers. D: Collect bees from tubular flowers using the insecticide bottle. E: Tilt the net to keep collected bees inside. F: Transfer collected bees into the bottle while still inside the net. Bees collected from the same flower species can share a bottle.

3. Specimen preparation

3-1. Tools

Writing materials, field notebook, forceps, wax paper, insect pins, insect boxes, desiccating tools (silica gel), shears, newspapers

3-2. Methods

Bees

Collected bees are pinned with insect pins according to their body size on the sampling day to produce dried specimens in good condition (Fig. 2A-H). Small individuals are glued to a card point and pinned through the broad end (Fig. 2C). The pinned specimens are completely dried in an oven at 60 °C for up to 3 days and then stored in insect boxes (Fig. 3). The data labels must be attached to each specimen individually (Fig. 4).

When it is not possible to return to the laboratory on the same day, follow the protocol below. Pinned specimens should be wrapped separately in wax paper according to the flower species they visited (Fig. 5). Each wax paper may contain up to 10 individuals, depending on the body size. Individuals that are too small to be pinned are simply wrapped in wax paper. The sampling date, name of the park, sampling point, and serial number of the plant species should be recorded on each wax paper. Wrapped bees are preserved in an airtight container for transport to the laboratory, with silica gel included to prevent mold. After arrival at the laboratory, transfer all samples onto a foam board and dry them in the same manner as unwrapped specimens. Finally, attach data labels to the specimens and store them in insect boxes.

Flowering plants

Plant samples are preserved in newspapers to produce dried specimens for taxonomic identification. For

details of the survey method for plant specimens, see *A Standardized Belt-Transect Survey Method*.

4. A proposed schedule

1st day: Survey for flowering plants in the park.

2nd–7th days: Bee sampling and specimen preparation*

* Depending on the volume of flowering plants and the number of inhabiting bees, continue sampling until the number of collected species at the park reaches saturation.

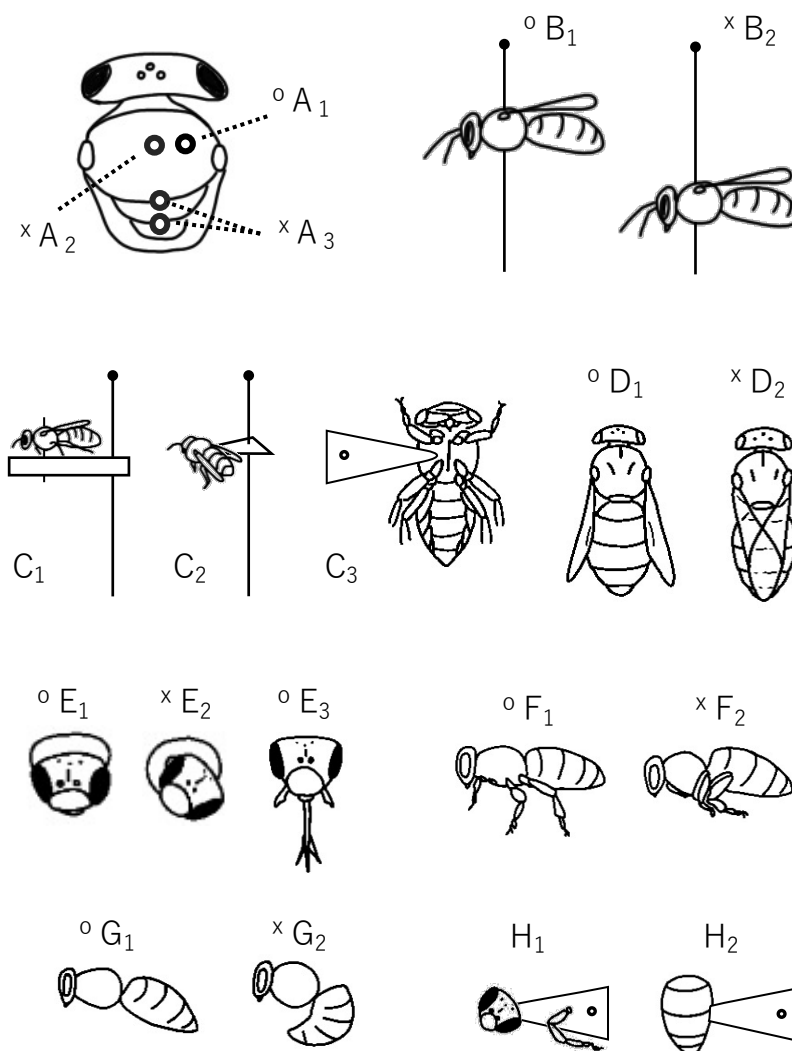


Fig. 2 Illustrations for desirable specimens. A: Position for pinning. Center of the right half of the mesoscutum is preferred (A_1). Other positions (A_{2-3}) may damage characters important for identification. B: Height of the body. Height depends on specimen size, but enough of the pin should always be exposed; 1) above the specimen to allow grasping without touching it with fingers, and 2) below, leaving space for at least three specimen labels. C: Double-mount method for small bees. A micro pin (C_1) or triangle card is used. The body is glued to the card point (C_{2-3}). D: position of wings. E: Position of head. The Mandibles are preferably opened (E_3). F: Position of legs. G: Position of abdomen. H: Broken parts are glued onto a card point.



Fig. 3 Specimens stored in insect boxes.

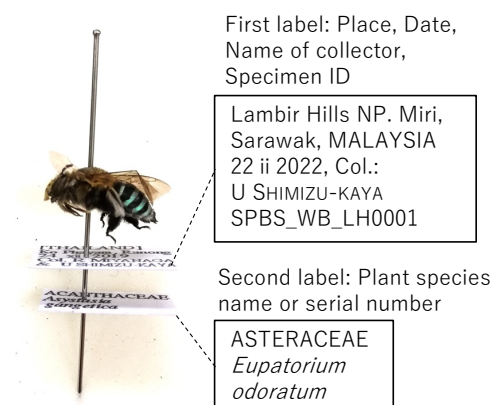


Fig. 4 Specimen with two data labels. A third label for taxonomic information is attached after species identification.

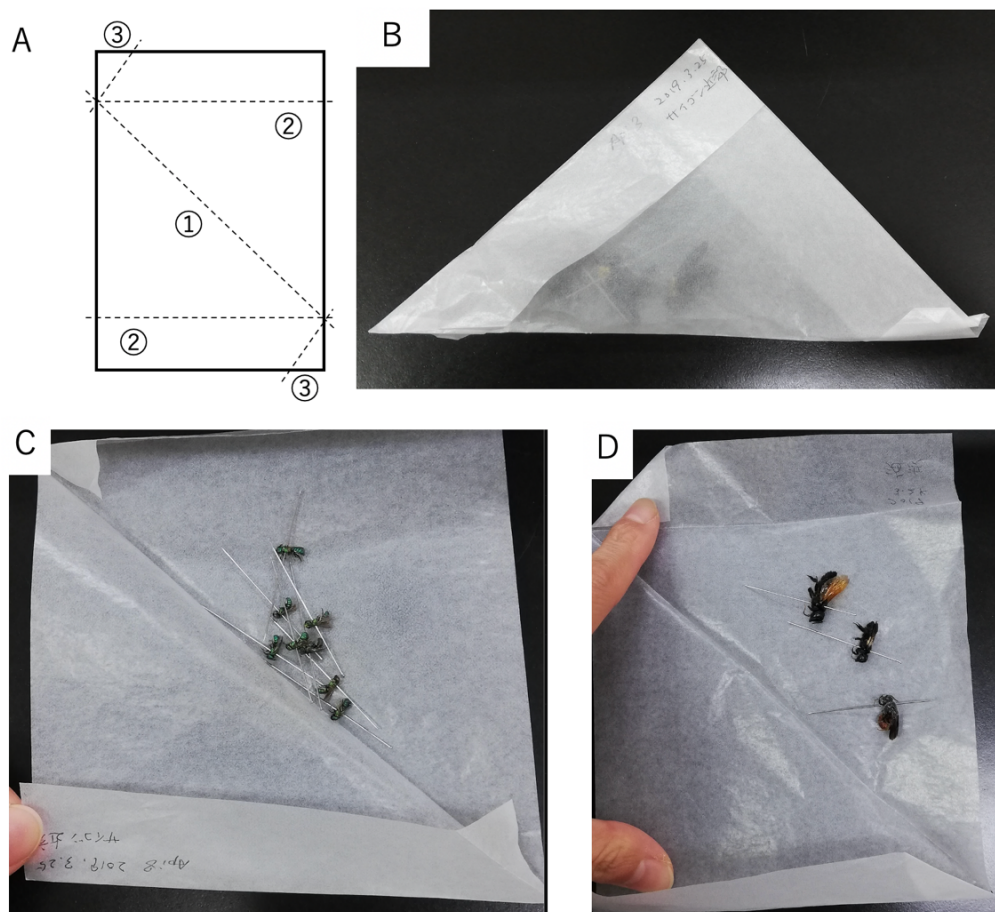


Fig. 5 Wrapped specimens. A: Design of the wax paper with folding order. B: Wrapped form. C: For small-sized bees. D: For moderate-sized bees.

5. References

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- Nagamitsu T, Inoue T (1994) Flower-visiting insects collected in lowland dipterocarp forests in Lambir Hills National Park, Sarawak. In: Inoue T, Hamid AA (eds.) *Plant Reproductive Systems and Animal Seasonal Dynamics—Long Term Study of Dipterocarp Forests in Sarawak*, pp. 142–145. Center for Ecological Research, Kyoto University, Ôtsu, Japan.
- Sakagami SF, Fukuda H, Kawano H (1974) Biofaunistic surveys of wild bees. Problems and methods, with results taken at Mt. Moiwa, Sapporo. *Seibutsu Kyozaï* 9: 1–60. [In Japanese with English summary]

4. Management of insect specimens

T. Itioka and H. Kawagoe

1. Importance of specimen management

Tropical rainforests harbor exceptionally high levels of biodiversity compared to other regions; therefore, they contain many undescribed species and taxa that are difficult to identify accurately based on morphological characteristics alone. As a result, the majority of organisms studied, or to be studied, in tropical rainforests present significant challenges for taxonomic differentiation. Naturally, when conducting scientific research on living organisms, accurate species identity of the studied individual is extremely important. One way to ensure accuracy and enable the verification of research results after publication is to properly store academic specimens. Furthermore, the results of current identification techniques and species determination may change owing to future advances in research technology and knowledge. Additionally, preserving these specimens is indispensable when comparing taxonomic groups with conspecifics or closely related species in other regions.

In research fields dealing with biodiversity, such as natural history and taxonomy, it is internationally recognized that specimens should be stored semi-permanently and made available for future research, as physical evidence (e.g., type and voucher specimens) of research results and as research materials for future research.

Furthermore, by linking, managing, and providing DNA samples extracted from specimens, alongside their research data and bibliographic information (Fig. 1), it becomes possible to advance future research and to expand upon previous research for a wide range of future applications.

Focusing on insect specimens which are central component of tropical rainforest biodiversity and often occur in large populations, this article describes a methodology for efficiently managing specimens and their associated data. This includes DNA obtained, analytical results, and other information obtained ensuring no data are lost throughout the process.

This method is currently being implemented in the management of insect specimens by the Research and Development Division (RDD) of the Forest Department of Sarawak (FDS). When cited in academic literature, this repository within the RDD is designated “Kuching Insectarium.”

2. Specimen storage

2-1. Digitizing the specimen data

For scientific use of insect specimens, the following data must be described on the label(s) attached:

Collection date

Collection site (latitude and longitude coordinates, if available)

Collector(s)

ID number of specimens (unique number different from all other specimens)

These data must be entered to the original database for the management of insect specimens following the preparation of the specimens and their associated data label. The original database should be handled by the curators of the insect collection.

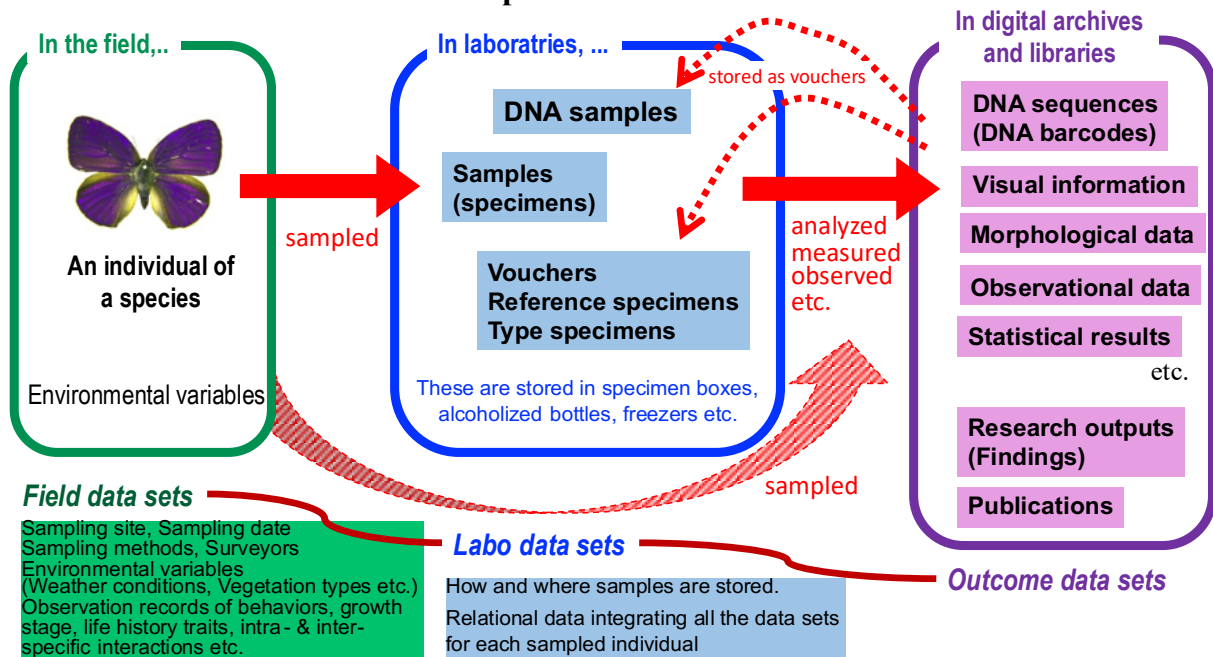
Other types of data, such as micro-environmental conditions, vegetation types, collection time, meteorological conditions, altitude, host plant, prey organisms, and symbiont species, should be written on additional label(s) or input to the original database or a particular relational database linked to the original database by the specimen ID number. The framework of the relational databases linked to the original database by specimen ID numbers is illustrated in Fig. 2-1.

2-2. Specimen sorting by taxonomic group

After attaching the data label and digitizing the data, the collected specimens should be sorted by taxonomic

groups (e.g., Order, Family, Genus) based on their morphology. If possible, the specimens should be sorted to the species level as a particular morpho-species (Fig. 2-2). After sorting, they should be stored in specific specimen boxes in their respective storage cabinets (Figs. 2-2, 2-3).

The management system for integrating various types of data on any information derived from an individual of a species.



The curators strictly keep the relations between three data sets for any samples.

Fig. 2-1 Schematic illustration of the structural organization of a relational database for the management of insect specimens.



Fig. 2-2 Specimen boxes containing lepidopteran specimens. Left: Specimens grouped by order level by collectors. Right: Specimens identified at the species level by taxonomists.

2-3. Storage of sorted specimens

After sorting, the specimens should be stored in specific boxes in a storage cabinet (Fig. 2-2, 2-3). The placement of specimens in storage is in accordance with the taxonomic system, making it easier for expert taxonomists studying a particular taxon to access them. Therefore, storing specimens from closely related taxa is advisable. For instance, closely related specimens should be stored in close proximity within a box, or if they

must be stored in separate boxes, they should be in boxes that are close together in a cabinet.

When a specimen is stored in a particular box, the cabinet ID number (Fig. 2-4) and the box ID number (Fig. 2-5) must be entered in the original database with the specimen ID or to the relational database linked to the original database by the specimen ID to avoid the loss of information on where the specimen is stored.

For more accurate species identification, taxonomic study, and future morphometrical and ecological studies, the stored specimens should be surveyed by expert taxonomists.

Type specimens should be stored in special cabinets, separately from others. Even specimens bearing labels attached at the time of collection that were assigned an ID may share that ID with other specimens within the collection; therefore, an additional ID unique to the insectarium is assigned.

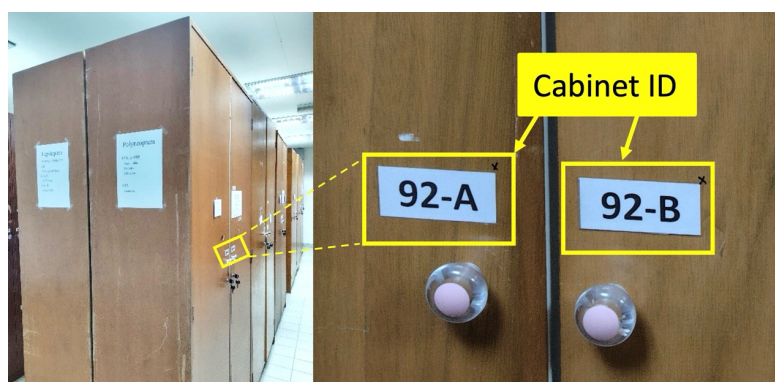


Fig. 2-4 Cabinets for storage of specimen boxes in Kuching Insectarium at RDD, FDS. Each cabinet is attached with its ID.



Fig. 2-3 Labels attached to a specimen. The data of the collection site (SARAWAK, Miri, Lambir), name of the project under which the collection was conducted, the method of collection (UV Light), height above the ground level ("T17" = 17 m), collection date, and specific collection code (G0137, code specific to the project indicates collection method details) are described in the upper label. The name of the storage site and specimen ID are written in the lower label.



Fig. 2-5 Specimen boxes in a cabinet. Each box is attached with its ID on the front side. The data of all the specimens stored in the box are linked to the Box ID.

3. Data management of each specimen

The abovementioned dataset of each specimen is entered into the database using a spreadsheet application, such as Microsoft Excel. In parallel with the entry of data on labels attached to specimens (Fig. 2-6, 2-7), any additional data, such as data on environmental and ecological factors, DNA samples and DNA sequences, results

of taxonomic studies, and bibliographic information of research articles using the specimens, should be entered into relational databases linked to the original database by specimen IDs (Figs. 2-1, 2-6).

The relational database system for managing specimen data and information in RDD is under development with FileMaker, a software used for cross-platform, low-code development, (Claris International Inc.). This platform facilitates the creation of a relational database system and the subsequent information addition following the creation of a key database of each specimen. Under PUBS, the FileMaker Pro 21.1. was installed on two computers at the Kuching Insectarium (RDD).

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Fig. 2-6 Example of sheets in two Excel files of data sets to be imported to the two FileMaker-formatted files as the relational databases, “R_DB” (left) and “BOX_ID” (right).



Fig. 2-7 Specimen photographed using a Keyence digital microscope. A: Photo taken from the dorsal side. B: Photo taken from the ventral side. C: Photo of the specimen label.

After inputting the collected data into an Excel file as mentioned above and checking its correctness, the data were imported into a FileMakerPro-formatted file.

The relational database for the management of the insect specimen collection comprises the following component database FileMakerPro-formatted files:

- 1) **R_DB**: This is the original (key) database for each insect specimen (Fig. 2-6) and includes at least the following data:
 - Specimen ID**
 - Collection date**
 - Collection site** (see explanation (3) below for standardization of location names),
 - Collector's name** (or **Title** of the project under which collection was conducted)
 - Box ID** (ID of the box in which the specimen was stored)
- 2) **BOX_DB**: This database indicates the cabinet in which the specimen box is stored (Fig. 2-6). It includes the following data:
 - Box ID**
 - Cabinet ID**
 - Order of the stored specimens** (optional)

(This database is linked to the original database of "R_DB" by **Box ID**)

- 3) **SITE_DB**: This database is used to register the standardized names of locations as collection sites. The location names of the collection sites are standardized to avoid using different location names to refer to the same location. The registered location names are linked to the collection site data recorded in the original database **R_DB**.

Collection site (standardized and registered when the data for each specimen is entered into **R_DB**)

- 4) **DET_DB**: This records and updates the species identity determined by an expert taxonomist for each specimen with a unique species code provided by the taxonomist. This is linked to **R_DB** by **Specimen ID** and linked to **TAXA_DB** by **Species code** (see below).

Specimen ID

Species code (to specify the identity of particular species that can be distinguished from others even without any particular name and to specify the updated species name or taxonomic category, determined by an expert taxonomist)

Determiner's name (to specify who identified the specimen and provided the species code)

Date of determination

- 5) **TAXA_DB**: This records and updates the species identification by an expert taxonomist for each specimen.

Determiner's name

Species code

Particular taxonomic names for various taxon classes (optionally including species name if specified)

These relational databases are directly or indirectly linked to each other through various ID types. Complete information on each species can be retrieved by checking the **Specimen ID**. The strict linkage of various types of scientific information for a particular specimen ensures the accuracy of the specimen's species identity when compared with other insect individuals worldwide, and the appropriate updating of taxonomic positions and categories, including species. The relational structure can be extended to the datasets of DNA samples extracted from the specimen, bibliographic data of published outputs from research derived from the specimen, and the content of manuals, guidebooks, and web-based platforms (Fig. 2-1).

In addition, a database for managing specimen loans for taxonomic studies by external researchers can be established, linked in a similar way to the original database by **Specimen ID**.

The datasets of a database can be compiled with those of other databases in various layouts (Fig. 2-8) using the functions provided by FileMaker.

4. Creation of image databases of insect specimens

Image databases of insect specimens are very useful for preserving their morphological information, future taxonomic and morphometric studies, and resources for the public, such as awareness activities, educational programs, content for eco-tours, nature watching tours, and nature experience tours. Photographs should be taken from various angles using depth compositing to capture morphological identifying characteristics. The dorsal, lateral, ventral, and head views of the specimen, along with the label, should be photographed for scientific use (Fig. 2-7).

High-resolution digital microscopes (Keyence microscopes were introduced into RDD and Sarawak Forestry Corporation (SFC) under PUBS) are suitable for capturing images of smaller insect specimens (approximately less than 30 mm in body length or span length of opening wings), whereas a single-lens reflex camera capable of capturing close-up shots is suitable for images of larger insect specimens (such as rhinoceros beetles and swallowtail butterflies).

The photo image data of each specimen can be linked to other relational databases, compiled with the datasets of the other databases, and displayed in various layouts (Fig. 2-8).

Insect specimen

Order

Family

Species

ID : **20063**

Species : **Catopsilia pomona**

Family : **Pieridae**

Order : **Lepidoptera**

Author name YEAR :

Collector : **Takuji Yamamoto**

Date : **20000729**

Site : **Lambir Hills National Park**

Local site :

Collection code :

Determiner :

Date of identification :

DNA :

Date of returned to RDD :

Box : **68.45** Cabinet : **45-B**

Memo :





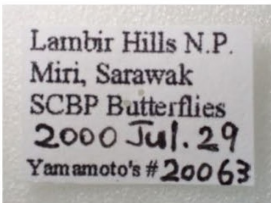






Fig. 2-8 An example of the FileMaker screen displaying compiled various data sets in the relational databases, including a photo image database.

5. Creating 3D models of insect specimens

H. Kawagoe

1. Introduction

Biological specimens are essential tools for organismal studies and are curated for public education in institutions such as museums. Preparation and preservation of insect specimens is challenging; their fragile morphology makes them susceptible to damage, necessitating strict management protocols. Such restrictions often limit physical access to specimens, leading to their inadequate utilization as research materials. To mitigate this limitation, 3D models of insect specimens were generated using photogrammetry, a technique for reconstructing 3D structures from images captured at multiple angles. This paper provides a detailed description of the methodology employed.

2. Tools

Camera
Tripod
White urethane foam
3DF Zephyr Lite (free version)

3. Photographing insect specimen

White urethane foam was used as a background, and the rotating platform constructed from urethane foam was secured (Fig. 1). To capture the entire specimen, it was incrementally rotated throughout the process (in 15° increments, capturing 24 images per full rotation). Photography was executed using focus stacking and ensuring that the entire specimen was captured. Reflections caused by the lighting were identified and mitigated by adjusting the illumination to minimize shadows. Two complete rotations were captured: the first from directly in front and the second from an angle slightly above and to the side (Figs. 2 and 3).

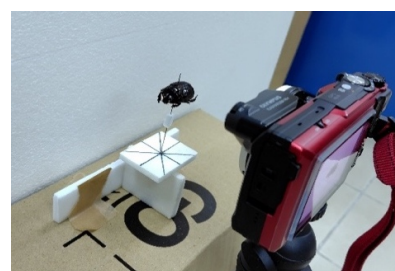


Fig. 1 Photographic equipment



Fig. 2 Photo shoot scene.

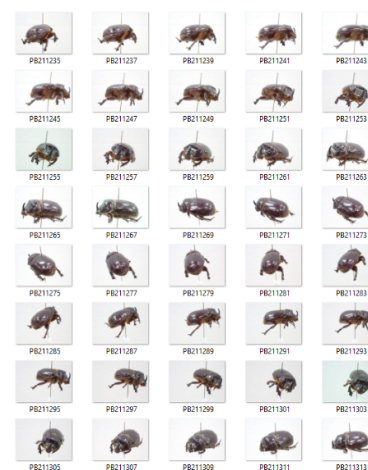


Fig. 3 Photos taken.

4. Generating 3D images using 3DF Zephyr Lite

4-1. Uploading photos

- “Workflow” was clicked at the top of the page, and “New project” was selected.
On the New Project Wizard screen, “Sparse” was selected.
Photos were uploaded from the “Photos selection” screen.
- The Free version of the 3DF Zephyr Lite can only load up to 50 photographs per sample.

4-2. Creation of sparse point clouds

We clicked “Next” on the previous screen.

On the “Camera orientation presets” screen, we selected:

Category > Surface scan

Preset > Deep

We clicked “Run”.

The screen shown in Fig. 4 indicates that a sparse point cloud has been successfully created. Photos where the “Reconstructed” field appeared in red with “No” indicated images where shooting location could not be determined. If this occurs frequently, accuracy can be compromised. In such cases, the previously mentioned presets must be adjusted before retaking the photos.

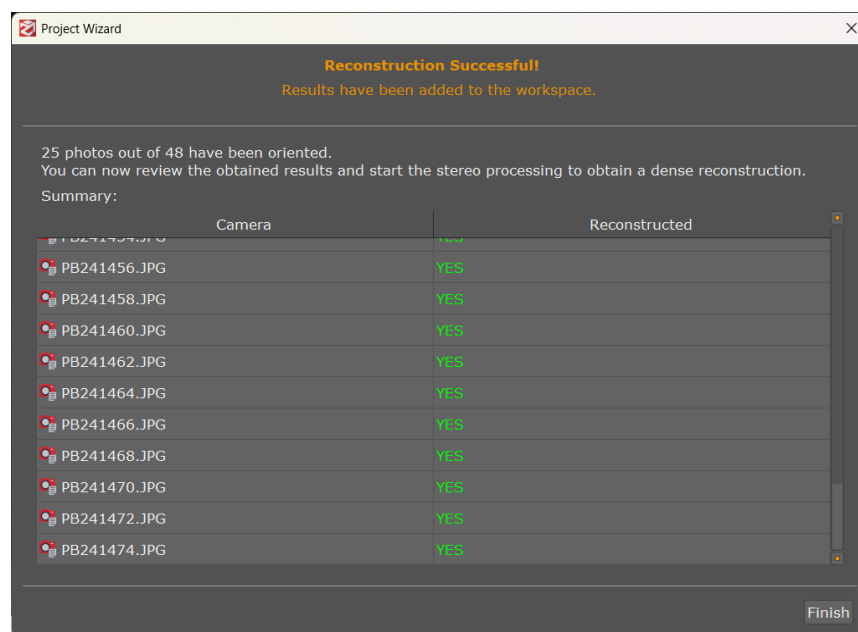


Fig. 4 Results of loading the photos.

4-3. Generate 3D models

- “Workflow” was clicked at the top of the page and “3D Model Generation” was selected
- Under “Dense Point Cloud creation” screen, we selected:
Category > Surface scan
Preset > High details
- Under “Surface reconstruction” screen, we selected:
Category > Surface scan
Preset > High details

We then clicked “Run”

After approximately 30 min of calculations, the 3D model was completed (Fig. 5).

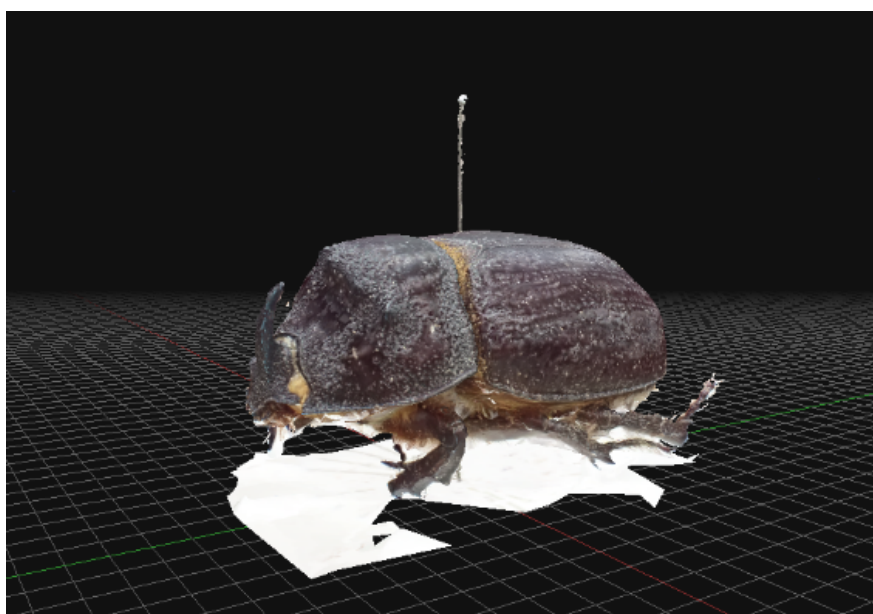


Fig. 5 Completed 3D model.

IV. Vertebrates

1. Reptile and amphibian diversity survey manual

Y. Kojima, I. Fukuyama and K. Nishikawa

1. Outline of the survey

- Target taxa
 - Squamata (lizards and snakes)
 - Testudines (tortoises and turtles)
 - Anura (frogs and toads)
 - Gymnophiona (caecilians)
- Sites
 - National Parks of Sarawak
- Objectives
 - Obtaining species inventories
 - Obtaining quantitative data on species abundance
 - Comparing the species composition patterns between the National Parks

Safety precautions

- There are venomous snakes in Sarawak. Identify the nearest hospitals that provide antivenom in advance of the survey. Carry a suction pump for venom extraction in case of a snakebite.
- Do not attempt to capture venomous or unidentified snakes unless absolutely necessary. Use a snake tong when handling venomous snakes. Never handle venomous or unidentified snakes by hand.
- If bitten by a venomous snake, immediately extract the venom using a suction pump and seek medical assistance to obtain antivenom. If possible, photograph the snake for identification.
- Before conducting a night survey, inspect the trails during daylight hours.
- Surveys should be conducted in parties of two or more persons whenever possible, particularly during night surveys.
- For night surveys, carry two or more flashlights and extra batteries.
- Wear gloves when handling amphibians to avoid disease transmission. Sanitize your hands if gloves are unavailable.

2. Field survey

- Objective: Obtain species inventory
- Materials: Torch light, headlamp, extra batteries, GPS, watch, thermometer, IC recorder, washing bag, plastic bag, writing utensils, notebook

Procedures:

- Before starting the survey, open a new page in the notebook and record the following information in the upper-left corner.
 - Date
 - Name of the National Park
 - Destination (trail name, etc.)
 - Name of researchers
 - Weather
 - Air temperature
 - Survey starting time
- Walk trails and visually search for reptiles and amphibians. Some tips include:

Watch the ground and vegetation carefully.

Turn over fallen logs or stones and check underneath (be careful of venomous snakes).

Return objects to their original position.

Check tree holes using a torch light.

Listen for the calls of frogs.

Frog eyes reflect light during the night.

Dig in the ground and scrape litter to search for fossorial species.

- Each time a species is found, take a photograph and record the species in the notebook.
- When species identification is difficult on-site, collect the individual (unless it may be a venomous snake).
- When the species may be a new record from the National Park, collect the individual (unless it may be a venomous snake).
- If there is a possibility of a venomous snake, only take pictures from a safe distance.
- Use tools to collect individuals if necessary.

Arboreal lizards (e.g., geckoes) may be swatted down with a fishing rod.

Terrestrial lizards (e.g., skinks) may be noosed or fished.

- Each time an individual is collected, photograph the site and record the following information in the notebook.

Collection time

ID number

Taxa (to the lowest level possible)

GPS coordinate

Habitat, microhabitat

Air temperature

Notes on behavior

- Collected individuals are kept in plastic bags (amphibians and small reptiles) or washing bags (large reptiles). Mark the bags with ID numbers. Avoid heat and pressure while carrying. Never put the bags under direct sunlight.
- When observing notable behaviors (e.g., feeding, reproduction, and defensive behaviors), take photos or videos and record the following information in the notebook. Collect the individuals when necessary (unless it may be a venomous snake).

Observation time

Taxa (to the lowest level possible)

GPS coordinate

Habitat, microhabitat

Air temperature

Details of the observation

- Upon finishing the survey, record the end time of the survey in the notebook.

3. Visual encounter survey (VES)

- Objective: Obtain abundance data on terrestrial and arboreal species.
- Materials: Torch light, headlamp, extra batteries, GPS, watch, thermometer, washing bag, plastic bag, writing utensils, notebook

Procedures:

- VES is conducted for approximately >40 person-h (e.g., 2 surveyors × 20 h) in each National Park.
- Select trails for VES. The trails should cover all main habitats in the National Park.
- Before starting the survey, open a new page in the notebook and record the following information in

the upper-left corner.

Date

Name of the National Park

Trail names

Name of surveyors

Weather

Air temperature

Time

GPS coordinate of the starting point

- Each time an individual is found, record the following information in the notebook.

Time

Taxa (to the lowest level possible)

Habitat

Height from the ground

- When species identification is difficult on-site, collect the individual (unless it may be a venomous snake) or take photographs.
- When collecting an individual, also record the following information.
 - ID number
 - GPS coordinates
- Collected individuals are kept in plastic bags (amphibians and small reptiles) or washing bags (large reptiles). Mark the bags with ID numbers. Avoid heat and pressure while carrying. Never put the bags under direct sunlight.
- Upon finishing the VES, record the following information.
 - Time
 - GPS coordinates of the end point

4. Pitfall survey

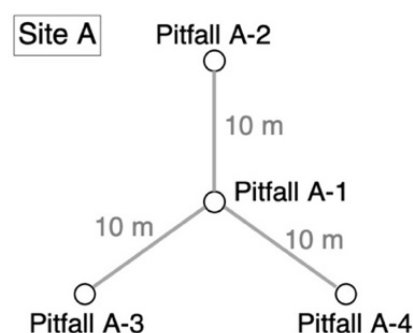
- Objective: Obtain abundance data on semi-fossorial and small terrestrial species.
- For logistical reasons, pitfall surveys are conducted only in National Parks with good access.

4-1. Installing pitfall traps

- Materials: Bucket with lid (12), plastic sheeting ($0.5 \text{ m} \times 10 \text{ m} \times 9$ pieces), stakes (approximately 75), cable ties or wire (about 200 pieces), shovel, knife, scissors, drill, measuring tape, gloves, GPS, writing utensils, notebook

Procedures:

- Choose 3 sites per National Park (site A–C).
- Decide the positions of pitfalls at each site. Pitfalls should be arranged as shown below.
- Record the GPS coordinates of each pitfall in the notebook.
- Prepare materials before starting work on site. Make a few holes (about $<3 \text{ mm}$ in diameter) in the bottom of the bucket using a drill. Cut a piece of plastic sheeting 50 cm wide and 10 m long and wrap it around the stake.
- Carry the materials and tools to the pitfall site.
- Bury the buckets at the predetermined position. The buckets function as pitfalls. Make sure the edges of the buckets are flush with or slightly lower than the ground.
- Make a ditch (about 5 cm deep) between the buried buckets. Set up the plastic sheeting using stakes as



supports. The sheeting functions as a drift fence.

- Fill the ditch with soil while pressing down the plastic sheeting. Ensure that the bottom edge of the sheeting is buried underground.
- Tie the plastic sheeting to the stakes with cable ties or wire.
- Mark each bucket with an identification number (e.g. A-1).

4-2. Checking pitfall traps

- Materials: Washing net, plastic bag, gloves, snake hook, torch light, writing utensils, notebook

Procedures:

- Check all pitfalls every morning.
- Be careful of dangerous animals (centipedes, scorpions, spiders, venomous snakes, etc.) in the pitfalls.
- Keep the traps functioning by carrying out the following actions:
 - Remove water and soil from the buckets.
 - Keep the edges of the buckets below the ground; fill in soil when necessary.
 - Check the drift fence. If there is a gap between the fence and the ground, backfill the fence.
- Record the following information in the notebook before starting the survey:
 - Date
 - Name of the National Park
 - Name of researchers
 - Weather
 - Time
- Each time a target animal is found in a pitfall, record the following information in the notebook:
 - Pitfall number
 - Taxa (to the lowest level possible)
 - Number of individuals per taxon (or sex, life stage).
- Collect the individual when species identification is difficult on-site, the individual is dead, or it may be the first record from the National Park. Other individuals should be released at the site after being recorded.

4-3. Removing pitfall traps

- Upon finishing the surveys, remove all pitfalls and drift fences (plastic sheeting and stakes).
- If the traps are to be reused, the buckets may remain, but the drift fences must be removed. In this case, all buckets should be completely sealed with their lids to prevent animals from falling in.

5. Species identification

Collected animals are identified according to the following references:

Das I (2025) *A field guide to the reptiles of Borneo*. Natural History Publications (Borneo).

Inger RF (1966) The systematics and zoogeography of the Amphibia of Borneo. *Fieldiana Zoology* 52: 1–402.

Inger RF, Stuebing RB, Grafe TU, Dehling JM (2017) *A field guide to the frogs of Borneo (3rd edition)*. Natural History Publications (Borneo).

Malkmus R, Manthey U, Vogel G, Hoffmann P, Kosuch J (2002) *Amphibians and reptiles of Mount Kinabalu (North Borneo)*. Koeltz Botanical Books.

Stuebing RB, Inger RF, Lardner B (2014) *A field guide to the snakes of Borneo (2nd edition)*. Natural History Publications (Borneo).

Taylor EH (1968) *Caecilians of the world*. University of Kansas Press.

6. Measurement

Materials: Tape measure, calipers, electronic balance, spring scale, tweezers, syringe, paper towels, writing utensils, notebook

- Measurements are conducted when necessary.
- After measurement, release animals as soon as possible at the site of capture (except when they are to be used for other studies).

6-1. Snakes

Stomach contents examination (only when necessary)

- Check for the presence of undigested stomach contents by digital palpation.
- When no contents are noticed, record as "no stomach contents" in the notebook.
- When contents are present, induce the snake to regurgitate by gently pushing them up with your fingers. Be careful not to let bones or spines get caught in the digestive tract. If you feel resistance, do not push hard; instead, push in the opposite direction.
- When the stomach contents are regurgitated, record the following information in the notebook:
 - Snake species
 - Snake ID
 - Stomach content taxon (to the lowest level possible)
 - Weight and size of the stomach contents
 - Direction in which the prey was swallowed (e.g., head-first)
- The stomach contents are fixed with 10% formalin or 70% ethanol and stored as specimens, or they may be returned to the stomach of the same individual.
- The snake may voluntarily swallow regurgitated prey if you put it in its mouth.
- If the snake does not swallow the regurgitated prey by itself, gently open the snake's mouth using tweezers, place the prey item in the mouth, and push it back to the digestive tract with the tweezers. Use your hands to send it down into the stomach. Be careful not to damage the snake's mouth or digestive tract. Do not attempt to force-feed large stomach contents, as this may cause injury to the snake.

Measurement

- Weigh the snake with a spring or electronic balance. If possible, measure the weight without stomach contents. If there are stomach contents, record "with stomach contents" in the notebook.
- Place the snake on the measuring tape and stretch its body to measure snout-to-vent length and tail length.
- Determine the sex based on the shape of the base of the tail or by popping. Record the sex in the notebook. If the sex is unknown, record "sex unknown".

6-2. Lizards

Stomach contents examination (only when necessary)

- Fill the syringe with water.
- Hold the lizard with one hand and carefully insert the syringe into the esophagus through the lizard's mouth with the other hand. Be careful not to insert the syringe into the trachea and send water into the lungs, or let the syringe penetrate the digestive tract, which may send water into the body cavity.
- Pump water through the esophagus and flush the stomach.
- Collect the stomach contents that the lizard regurgitates with the water.
- If the lizard does not regurgitate the stomach contents, repeat the above procedures.
- Stomach contents are fixed in 70% ethanol and identified under a microscope in the laboratory.

Measurement

- Weigh the lizard with an electronic balance.

- Measure snout-to-vent length, tail length, head width, and head length with calipers.
- Determine the sex based on the shape of the base of the tail or by popping. Record the sex in the notebook. If the sex is unknown, record "sex unknown".

6-3. *Frogs*

Stomach contents examination (only when necessary)

- Hold the frog in the supine position (upside down) with one hand, and open its mouth using tweezers held in the other hand.
- While gently poking the back of the throat with tweezers, push up the belly with the thumb of the hand holding the frog until the frog flips its stomach.
- Collect stomach contents from the flipped stomach with tweezers.
- Put the flipped stomach back into the mouth.
- When it is difficult to make the frog regurgitate (e.g., in the case of large frogs), anesthetic (MS222) may be used.
- Stomach contents are fixed in 70% ethanol and identified under a microscope in the laboratory.

Measurement

- Weigh the frog with an electronic balance.
- Measure snout-to-vent length, head width, and head length with calipers.
- Determine the sex based on the presence or absence of nuptial pads, vocal sac, and body size. If the sex is unknown, record "sex unknown".

7. Making specimens

Materials: 99% ethanol, 70% ethanol, 10% formalin, anesthesia (pentobarbital [for reptiles], chlorotone [for amphibians]), plastic box with the lid (for formalin), syringe and needle (for formalin), gloves (for formalin), scissors, tweezers, sample tubes, specimen jars, sample labels, water-resistant paper, pencils, needles, thread, paper towels, camera, scale, color samples, white background

- Tissue samples for DNA extraction are stored in 99% ethanol, and the remaining body is fixed in formalin and then stored in 70% ethanol.
- Make labels for tissue samples by cutting pieces of water-resistant paper and writing sample numbers on them with a pencil. Make sure that the numbers match the sample numbers of the bodies.
- If necessary, photograph animals in a living state.
- Euthanasia should be carried out by overdose of anesthesia.
- Place the specimen, sample label, scale, and color chart on a white background and take photographs of the dorsal, ventral, and lateral views.
- Collect a tissue sample (e.g., 2-mm square of the liver) using scissors and tweezers cleaned with 70% ethanol. Minimize the incision and clean the instruments each time.
- Put the tissue in a sample tube with 99% ethanol and a sample label inside. Tissue samples should be stored in a freezer or refrigerator (or at room temperature if it is difficult to keep them cool).
- Attach the sample label to the body.
 - Lizards with four limbs: Tie to the waist.
 - Frogs: Tie to the hind leg (the knee).
 - Snakes, caecilians, and limbless lizards: Sew to the skin at the incision from which the tissue sample was taken.
- Fix the body with formalin. Wear rubber gloves when using formalin.
 - Lay paper towels in a plastic box and pour 10% formalin into it.
 - Place the specimen and arrange the posture according to the figure below.
 - For reptiles, inject 10% formalin with a syringe into several positions to fill the abdominal cavity and tail.

- For male reptiles, inject 10% formalin at the base of the tail to inflate the hemipenes.
- Cover the specimens with paper towels and pour 10% formalin over them.
- Leave the specimen for several hours to half a day (amphibians) or half a day to 1 day (reptiles) at room temperature.
- After fixing, soak the specimens in running water for 1 day (amphibians) or 1–2 days (reptiles) to remove formalin. Transfer the specimens to 70% ethanol and store at room temperature.



8. Compiling data

The data for each National Park are compiled as follows and used for comparison.

Species inventory

- Each researcher lists the species they observed during the field survey and VES.
- Combine the lists and the results of the pitfall survey to obtain species inventory.

Abundance data from VES

- Each researcher summarizes their own results from VES in an Excel file.
- In the file, each row represents one species, and each column represents the number of individuals seen in one VES.
- Also enter the following records for each VES in the respective rows:
 - Date of VES
 - Trail name
 - Habitat
 - Weather
 - Names of the surveyors.
- Combine the results from all surveyors.

Abundance data from the pitfall survey

- The results from the pitfall survey are summarized in an Excel file.
- In the file, each row represents one species. Separate sex and/or growth stage into different rows when they are distinguished. Each column represents the number of individuals for 1 day.
- Enter the weather in another row.

9. Model plan

Below is an example schedule for a 12-day fieldwork period conducted by two persons.

Day 1: Field survey, check candidate trails for VES, check candidate locations for pitfall survey
Days 2–4: Field survey, check candidate trails for VES, install pitfalls
Days 5–10: Field survey, VES, pitfall survey
Day 11: Preparing specimens, removal of pitfalls
Day 12: Spare day
Measurements and specimen preparation are conducted whenever necessary.

10. References

Refer to the following references when necessary:

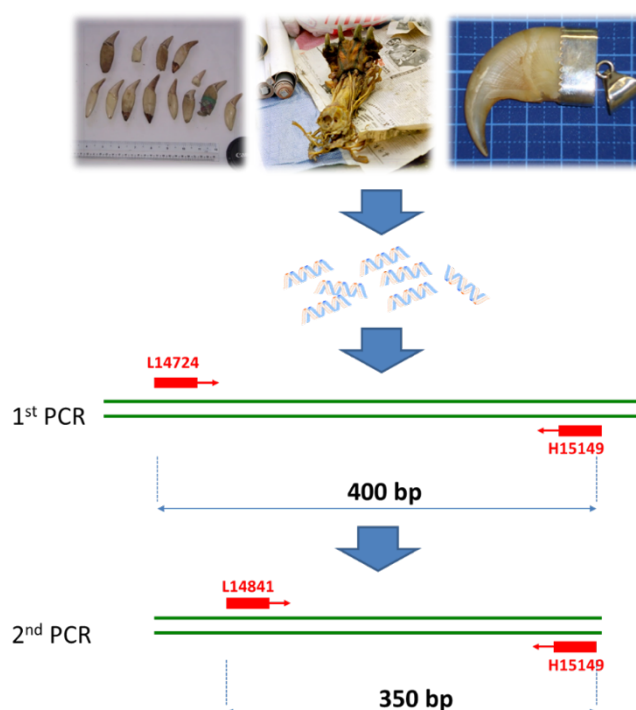
- Dodd CK (ed.) (2016) *Reptile ecology and conservation: a handbook of techniques*. Oxford University Press, Oxford.
- Heyer R, Donnelly MA, Foster M, McDiarmid R (eds.) (2014) *Measuring and monitoring biological diversity: standard methods for amphibians*. Smithsonian Institution, Washinton DC.
- Lips KR, Reaser JK, Young BE, Ibañez R (2001) *Amphibian monitoring in Latin America: a protocol manual/Monitoreo De Anfibios En América Latina: Manual De Protocolos*. Herpetological Circular 30. Society for the Study of Amphibians and Reptiles.
- Wilkinson JW (2015) *Amphibian survey and monitoring handbook*. Pelagic Publishing Ltd.

2. Species identification for confiscated wildlife body parts

M. Onuma

Overview

Mitochondrial DNA and cytochrome b genes will be sequenced to identify the species of confiscated specimens. L14724 and H15149 (Irwin et al. 1991) will be used as primers for PCR. The semi-nested PCR method will be carried out if no amplification of the PCR product is observed with this primer set. L14724 and H15149 (Irwin et al. 1991) are used for the first PCR, whereas L14841 and H15149 (Kocher et al. 1989) are used for the second. The reagents for amplification are prepared using KOD-Plus-V.2 (Toyobo). The obtained sequence is subjected to homology analysis. The species with the most homologous sequence is determined to be the species of origin of the confiscated items.



References

- Irwin DM, Kocher TD, Wilson AC (1991) Evolution of the cytochrome b gene of mammals. *Journal of Molecular Evolution* 32:128–44.
- Kocher TD, Thomas WK, Meyer A, Edwards SV, Peabo S, Villablanca FX, Wilson AC (1989) Dynamics of mitochondrial DNA evolution in animals: amplification and sequencing with conserved primers. *Proceedings of the National Academy of Sciences of the United States of America* 86: 6196–6200.

1. DNA extraction

Several DNA extraction methods have been reported, and many commercial kits are available. We recommend the following commercial kits:

Soft tissues and blood (mammal and bird): DNeasy Blood & Tissue Kit (QIAGEN;
<https://www.qiagen.com/us/products/discovery-and-translational-research/dna-rna-purification/dna-purification/genomic-dna/dneasy-blood-and-tissue-kit/>)

Hard tissues (claw, bone, teeth etc.) and animal hair: ISOHAIR (NIPPON GENE;
https://www.nippongene.com/english/tds/tds_isohair-e.pdf)

The procedures for DNA extraction have been described in Appendices 1 (DNeasy Blood & Tissue Kit) and 2 (ISOHAIR).

2. PCR reaction

Primers

L14724: 5' -CGAAGCTTGATATGAAAAACCATCGTTG-3'
 H15149: 5' -AAACTGCAGCCCCTCAGAATGATATTTGTCCTCA-3'
 L14841: 5' -AAAAAGCTTCCATCC AACATCTCAGCATGATGAAA-3'

PCR reaction mixture

(KOD PLUS-VER.2; https://www.toyobo-global.com/seihin/xr/lifescience/products/pcr_001.html)

First PCR

D.W.	34.4µl
10× Buffer for KOD-Plus- Ver.2	5 µl
2mM dNTPs	5 µl
25mM MgSO ₄	3 µl
L14724 (50µM)	0.3 µl
H15149 (50µM)	0.3 µl
KOD-Plus- (1.0U/µl)	1 µl
DNA extract	1 µl

The protocol for the first PCR

94°C 2 min
 ↓
 (98°C 10 s _ 50°C 30 s _ 68°C 1 min) × 35 cycles
 ↓
 4° C ∞

Electrophoresis

Gel electrophoresis is conducted to visualize PCR products. 2% agarose gel is required to identify the products of L14724 and H15149.

If a PCR product is identified at around 400 bp, proceed with sequencing. If no PCR products are identified, perform a second PCR.

Second PCR

D.W.	34.4µl
10× Buffer for KOD-Plus- Ver.2	5 µl
2mM dNTPs	5 µl
25mM MgSO ₄	3 µl
L14841 (50µM)	0.3 µl
H15149 (50µM)	0.3 µl
KOD-Plus- (1.0U/µl)	1 µl
The reaction mixture of the first PCR	1 µl

The protocol for the second PCR

94°C 2 min

↓

(98°C 10 s _ 50°C 30 s _ 68°C 1 min) × 35 cycles

↓

4° C ∞

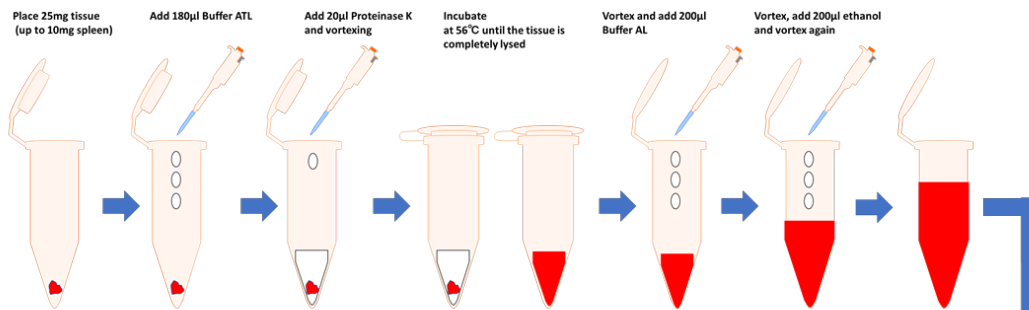
Electrophoresis

Gel electrophoresis is conducted to visualize PCR products. 2% agarose gel can be used to identify the products of L14841 and H15149. If the PCR product is identified at around 350 bp, proceed with sequencing.

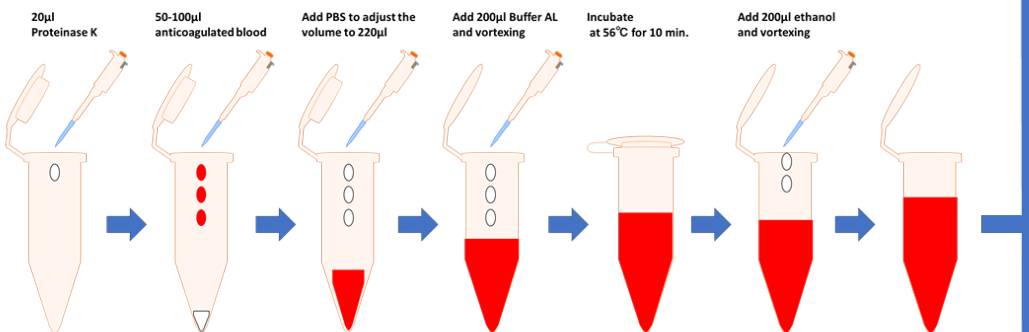
Appendix 1

Preprocessing for DNA extraction using DNeasy Blood & Tissue Kit (QIAGEN)

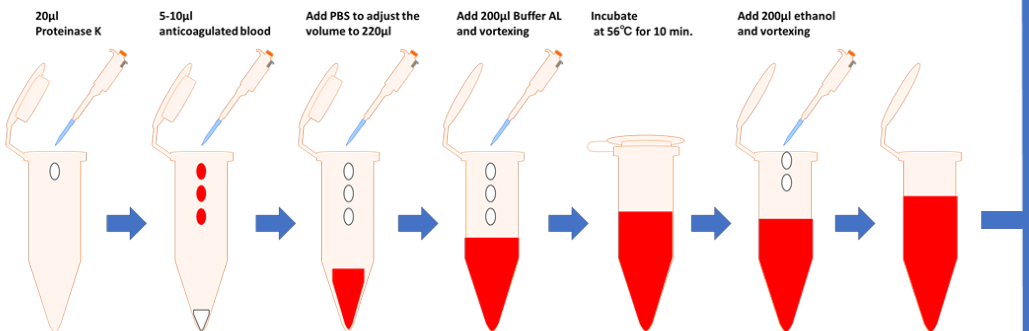
Tissue sample



Blood sample (Mammal)



Blood sample (Bird)

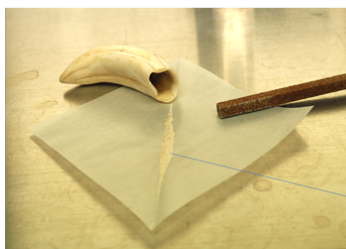


**Spin column purification
(Follow QIAGEN's instruction)**

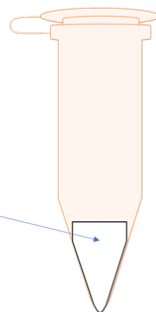
Appendix 2

DNA extraction using ISOHAIR(NIPPON GENE) from hard tissues (bone, teeth, crow etc.)

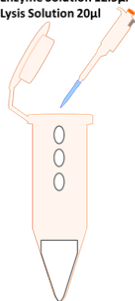
1, File hard tissues (bones, Teeth, crows) into a powder



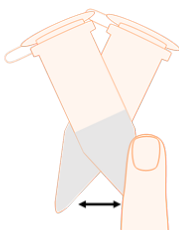
2, Transfer the powder into a new tube



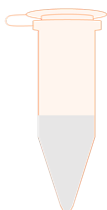
3, Add
Extraction Buffer 500µl
Enzyme Solution 12.5µl
Lysis Solution 20µl



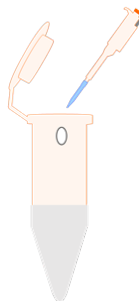
4, Mix by tapping tube
several times with a finger



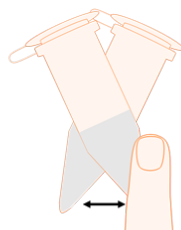
5, Incubate, 55°C, 20 min



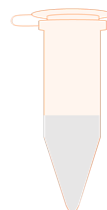
6, Add
Enzyme Solution 12.5µl



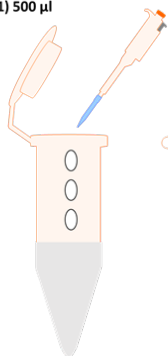
7, Mix by tapping tube
several times with a finger



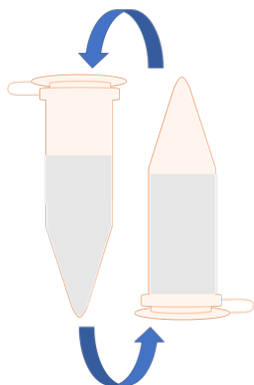
8, Incubate, 55°C, for overnight



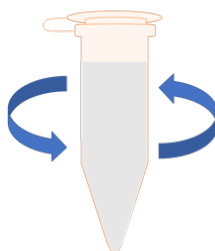
9, Add
Phenol/chloroform/isoamyl alcohol
(25:24:1) 500 µl



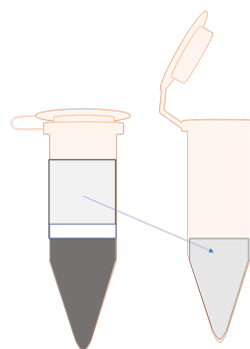
10, Mix gently by inversion
for 5min.



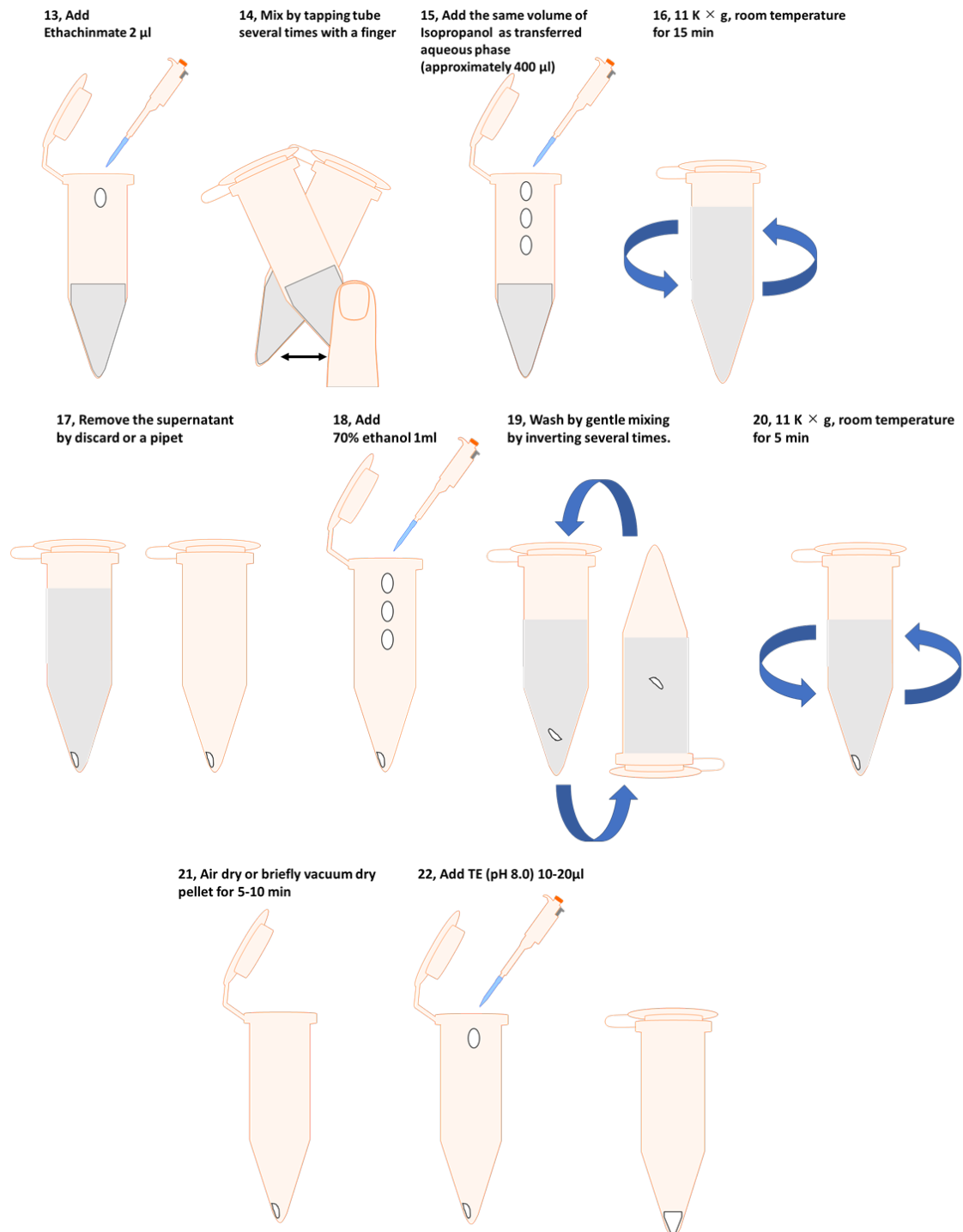
11, 11 K × g, room temperature
for 5 min



12, Transfer aqueous phase
to a new tube



Appendix 2 (continued)



V. DNA-Sequencing

1. Preparation of the DNA library for NGS

H. Sato and T. Mizuno

For Safe Molecular Experiments

1. Wear Experimental Protective Gear

Always wear personal protective equipment in the laboratory. Before entering, put on a long-sleeved lab coat, closed-toe shoes, and safety glasses. Tie back long hair.

2. Dispose of Waste Safely

When discarding laboratory reagents and chemicals, confirm their hazards. If the materials contain hazardous substances, do not pour them down the sink. Rather, place them in the designated waste container. Handle waste disposal in accordance with all applicable laws and regulations.

3. Use and Inspect Equipment Properly

Always read the manual for the laboratory equipment you use and ensure its proper operation. Furthermore, perform regular inspections according to the manual. Proper use and management are especially critical for centrifuges and autoclaves, as their improper handling can lead to serious accidents.

General Considerations for Molecular Experiments

1. Clean laboratory tools and glassware thoroughly.
2. Wipe the workbench with diluted bleach and/or 70% ethanol.
3. Maintain reagents and samples at the appropriate temperatures. Use cooling blocks as needed and promptly return reagents and samples to the refrigerator or freezer after use.

DNA extraction protocol

• DNA extraction from plant and fungal tissue (CTAB method)

0. Set the temperature of the heating block to 55°C. Prepare the same number of 2-ml and 1.5-ml sterile tubes as the number of samples and assign sample numbers to the tubes. Cool isopropanol and 70% ethanol at -20°C.
 1. Add 100µl of mercaptoethanol to 20ml of 2×CTAB extraction buffer.
※ Mercaptoethanol can be omitted.
 2. If samples are stored in ethanol, dry them for 5–10 min to remove the ethanol. Transfer the sample (up to 5mm³ or 1cm² leaf piece) and a 5mm zirconia ball to a 2-ml tube.
 3. Smash the samples with TissueLyser (30/s for 2 min). If you cannot smash it well, freeze the sample tubes in -20°C and then smash them with TissueLyser. (However, there is a risk of tube breakage.)
※ **Place an equal number of tubes on right and left racks of TissueLyser to maintain balance.**
- (Steps 4 and 5 can be skipped if the sample contains minimal impurity.)
4. Add 500 µl of wash buffer and invert the tubes to mix. Centrifuge at 5,000 rpm for 5 min at 4 °C. Discard the supernatant.
 5. Repeat step 4 an appropriate number of times, depending on the color and viscosity of the solution.
 6. Add 500µl of CTAB buffer and incubate at 55°C for 20 min (*1). Invert the tubes a few times during incubation. Briefly centrifuge the tubes.
 7. Add 500µl of CIA solution (chloroform : isoamyl alcohol = 24 : 1) and vortex it (*2). Centrifuge it for 5 min (20°C, 15,000rpm).
 8. Transfer approximately 300µl of the supernatant to a new 1.5ml tube using a 200µl pipette. (DO NOT extract the lower layer. It is not necessary to extract all 300 µL of the supernatant.)
 9. Add 300µl of -20°C isopropanol to the supernatant and stir well (*3). Centrifuge for 10 min (4°C, 15,000rpm).
 10. Remove the supernatant without disturbing the pellet of precipitated DNA.
 11. Add 500µl of -20°C 70% ethanol and stir. Centrifuge for 5 min (4°C, 15,000rpm) (*4).
 12. Decant the ethanol (or remove it with a pipette). Centrifuge for 1 min (4°C, 15,000rpm) to place the DNA pellet at the bottom. Remove the residual ethanol by drying.
 13. Add 50µl of TE buffer and dissolve the DNA pellet. If you are unable to dissolve the pellet, warm the tube at 55°C. This DNA solution is used as a sample for subsequent PCR (*5).
- (*1) CTAB extraction buffer breaks cell, nuclear, chloroplast, and mitochondrial membranes to lyse the DNA inside at 55°C. Mercaptoethanol acts as an antioxidant.
- (*2) Chloroform acts as a protein-denaturing agent and separates proteins from DNA. Isoamyl alcohol acts as a foam suppressant.
- (*3) As DNA is a polymer colloid, it is precipitated by adding alcohol and salt (salting-out). This process is known as ethanol/isopropanol precipitation.
- (*4) 70% ethanol is added to remove residual salt.
- (*5) TE buffer is more suitable for DNA storage than sterile water because the EDTA in TE buffer inhibits DNase activity.

Preparation of DNA extraction reagents

0.1M HEPES (pH 8.0)

HEPES 2.38g

Pure water 80ml

NaOH → adjust to pH 8.0 (with a pH meter)

Pure water, up to 100 ml

※ As it is heat-sensitive, do not autoclave. Because it is also light-sensitive, completely wrap the bottle with aluminum foil and store it in the dark.

Wash buffer

0.1M HEPES (pH 8.0) 100ml

Mercaptoethanol 2ml (extract using a syringe)

PVP 1.0g

Ascorbic acid 0.88g

※ Prepare immediately before use.

2×CTAB extraction buffer: 500ml

CTAB: 10g

NaCl: 41.0g

1M Tris-HCl (pH = 8.0): 50ml

EDTA -2Na: 3.7g (0.5M EDTA, 20ml)

Pure water: dilute the reagents above up to 500ml.

CIA(chloroform : isoamyl alcohol = 24 : 1): 250ml

chloroform: 240ml

isoamyl alcohol: 10ml

TE (Tris-EDTA): 500ml (autoclave)

1M Tris-HCl buffer: 5ml

0.5M EDTA: 1ml

Pure water: 494ml

● DNA extraction from insects (Chelex method)

0. (Preparation of 10% Chelex-TE solution) Mix 2g of Chelex resin and 20ml of 1×TE buffer.

※ Change the amount of Chelex and TE buffer depending on the number of samples.

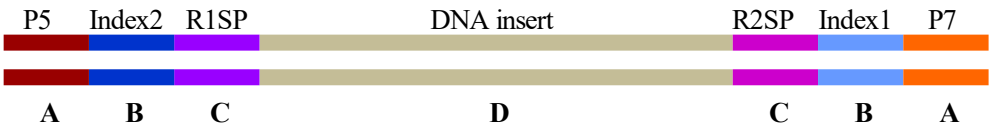
1. If a sample is old or dirty, wash it with 500μl of TE buffer in a 1.5ml tube and vortex it. Next, transfer the sample to new 1.5ml tube.
2. Add 100μl of the Chelex-TE solution and 5μl of Proteinase K into the tube. Then, crush the sample with a pestle or a plastic stick in the tube. Because Chelex resin settles easily, gently mix the tube to resuspend the Chelex resin before transferring it.
3. Incubate the tube at 56°C for at least 3 h.
* Longer extraction time can increase yield.
4. Incubate the tube at 99°C for 3 min.
5. Centrifuge the tube at high speed (10,000–12,000g) for 3 min and then transfer the supernatant to a new tube. (No need to transfer the whole solution. Be careful not to extract the Chelex resin.)

Polymerase chain reaction (PCR)

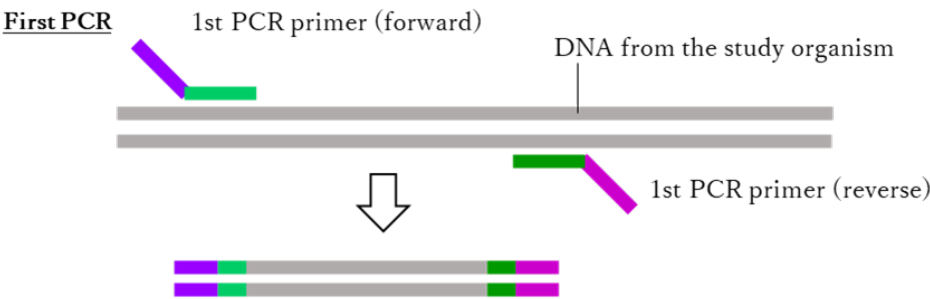
There are several methods for the preparation of DNA libraries for next-generation sequencing. Here, we describe a DNA library preparation method using a two-step tailed PCR, designed for sequencing on the Illumina MiSeq platform.

The first PCR amplifies the target DNA region from the study organism. The second PCR adds the adapter sequences required for MiSeq sequencing, sample-specific unique index sequences, and sequencing primer binding sites.

Schematic representation of the MiSeq library structure



A: Illumina adapter sequences (P5 & P7) for binding to the flow cell
B: Index sequences
C: R1 & R2 sequencing primer binding sites
D: DNA region of interest from the study organism



1st PCR forward primer (*rbcLa**)

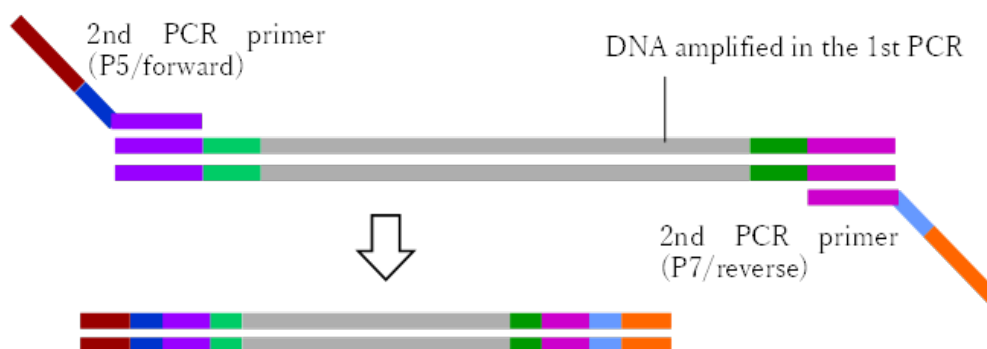
5' -CGCTCTTCGATCTCTGNNNATGTCACCACAAACAGAGACTAAAGC-3'
The sequence of the target DNA region

1st PCR reverse primer (*rbcLa**)

5' -TGCTCTTCGATCTGACNNNGTAAAATCAAGTCCACRCG-3'
The sequence of the target DNA region

* Kress WJ et al. (2009) Plant DNA barcodes and a community phylogeny of a tropical forest dynamics plot in Panama. *Proceedings of the National Academy of Sciences of the United States of America*, 106, 18621–18626.

The target-specific sequence of the first PCR primers should be modified according to the study organism and the target DNA region (the primers shown above are designed for the plant *rbcL* region). Four primers containing 0–3 random bases (N) between the target-specific and tail sequences were mixed and used to improve sequencing quality. The first primers in the figure contain three random bases. This increases base diversity in the initial sequencing cycles, thereby improving base-calling accuracy.

Second PCR

P5 2nd primer

AATGATACGGCGACCACCGAGATCTACACGGCCATATACACTCTTTCCCTACACGACGCTCTTCCGATCTCTG
 index

P7 2nd primer

CAAGCAGAAGACGGCATACGAGATAGAGACACGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTGAC
 index

Use sample-specific unique index sequences for the second PCR. This enables the assignment of sequencing reads to their corresponding samples based on the index sequences during downstream data analysis.

First PCR (amplification of the target DNA region)**• Preparation of the reaction solution (total volume: 10µl)**

1. Mix all reagents except for the DNA template in a 1.5ml tube (pre-mix). Some loss is inevitable during dispensing. Therefore, prepare a slightly larger volume of the pre-mix than required.
2. Dispense 9µl of pre-mix into each tube.
3. Add 1µl of the DNA template into the tube and mix by pipetting. (Change tips each time)
4. Cap the tube and centrifuge briefly.

Standard pre-mix formulation for KOD FX Neo (TOYOBO Co., Ltd.)

Reagents	N = 1	Final concentration
Water	0.6 µl	
2× PCR buffer	5.0 µl	1 ×
dNTPs (2mM)	2.0 µl	0.4 mM
KOD FX Neo (1 U/µl)	0.2 µl	0.02 U/µl
1 st PCR Primer F (5µM)	0.6 µl	0.3 µM
1 st PCR Primer R (5µM)	0.6 µl	0.3 µM
DNA template*	1.0 µl	

* Diluting the DNA template approximately 10-fold may improve PCR amplification.

• Thermal cycler settings for the first PCR

The settings shown below are the standard settings for the KOD FX Neo kit. Modify the settings according to the manufacturer's instructions when using other PCR kits.

Determine the annealing temperature based on the primers used.

Step description	Temp.	Duration	Number of cycles
Hot start	94°C	2 min	1
Denaturation of the DNA double strand	98°C	10 s	35
Annealing of primers	depends	30 s	
Elongation by DNA polymerases	68°C	30 s	
Final elongation	68°C	5 min	1
Hold for storage	10°C	∞	1

Second PCR (addition of index, adapter, and sequencing primer binding site)

● **Preparation of the reaction solution (total volume: 10µl)**

1. Mix all reagents except for the DNA template and primers in a 1.5ml tube (pre-mix). Prepare a slightly larger volume of the pre-mix than required.
 2. Dispense 7.8µl of pre-mix into each tube.
 3. Add 1.2µl of a mixture of forward and reverse primers (unique index pairs of forward and reverse primers) into the PCR plate using an 8-channel pipette. (Change tips each time)
- ※ Be sure to accurately record which index combination was used for each sample. If this information is unclear, it will be impossible to match samples with their corresponding sequencing reads, which will render the data unusable.
4. Add 1µl of the DNA template into the plate. (Change tips each time)
 5. Seal the plate and centrifuge briefly.

Standard pre-mix formulation for KOD FX Neo (TOYOBO Co., Ltd.)

Reagents	N = 1	Final concentration
Water	1.2 µl	
2× PCR buffer	5.0 µl	1 ×
dNTPs (2mM)	2.0 µl	0.4 mM
KOD FX Neo (1 U/µl)	0.2 µl	0.02 U/µl
2nd PCR Primer mix F&R (2.5µM each)	1.2 µl	0.3 µM
DNA template	1.0 µl	

● **Thermal cycler settings for the 2nd PCR**

The settings shown below are the standard settings for KOD FX Neo. Modify the settings according to the manufacturer's instructions when using other PCR kits. The annealing temperature for the second PCR is always 60°C.

Step description	Temp.	Duration	Number of cycles
Hot start	94°C	2 min	1
Denaturation of double-strand DNA	98°C	10 s	12
Annealing of primers	60°C	30 s	
Elongation by DNA polymerases	68°C	30 s	
Final elongation	68°C	5 min	1
Hold for storage	10°C	∞	1

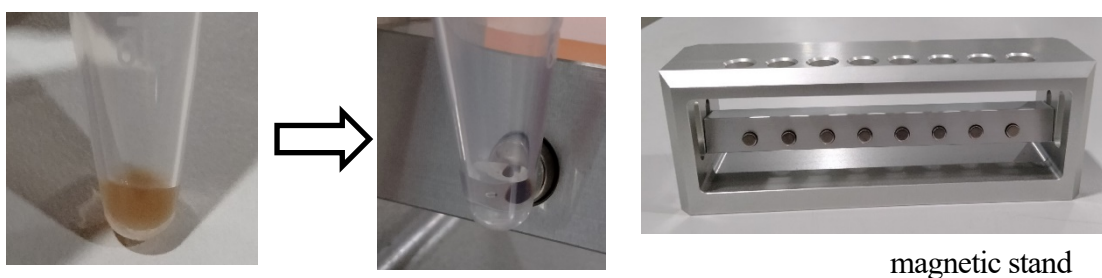
After PCR, add equal amounts of each PCR product into a tube (DNA library for subsequent purification and NGS analysis). If the concentration of PCR products is extremely uneven, adjust their proportion.

Purification and size selection of the DNA library

To obtain high-quality results from MiSeq analysis, proper DNA purification and size selection are essential, especially the removal of short non-target fragments (e.g., PCR-derived primer dimers) and excessively long DNA fragments that cannot be sequenced by MiSeq. After pooling the second PCR products into a single tube, excessively short and long DNA fragments can be removed using the following two procedures:

• Purification and size selection of the DNA library by AMPure XP

1. Mix the library (2nd PCR product mixture) and AMPure XP in a 1.5 ml tube at a 1:0.8 ratio.
2. Mix the sample by pipetting 10 times and leave it for 5 min (DNA fragments bind to magnetic beads).
3. Place the tube on a magnetic stand, leave it for 2 min or until the solution becomes clear, and remove the supernatant.
(* Impurities are contained in the supernatant, whereas DNA fragments are retained on the magnetic beads.)

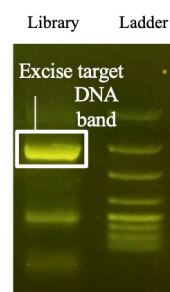


4. Add a larger volume of 70% ethanol than that of the DNA library–AMPure mixture to the tube on the magnetic stand, leave it for 30 s, and then remove the supernatant.
(* This is a washing step. A larger volume of 70% ethanol is required to ensure complete removal of the remaining supernatant on tube wall.)
5. Repeat Step No. 4.
6. Remove the tube from the magnetic stand, add 40 µl of pure water, and dissolve the magnetic bead pellet by pipetting 10 times. Then, leave it for 5 min.
(* The DNA fragments move from the magnetic beads to pure water in this step)
7. Place the tube onto the magnetic stand and leave it for 1 min.
8. Transfer the supernatant to a new tube. This supernatant contains purified DNA fragments.

• DNA size selection by gel excision

Separate the DNA library by electrophoresis and excise the DNA fragments of the target size using a clean scalpel or forceps.

Extract the DNA fragments from the excised agarose gel. Various commercial kits are available for this purpose (e.g., FavorPrep™ Gel/PCR Purification Kit (FAVORGEN)).



Control of the DNA library concentration

After size selection, accurate adjustment of the DNA library concentration is critical for MiSeq. Because sequencing depends on the number of adapter-ligated DNA molecules rather than fragment length, both the average fragment length and DNA mass concentration must be determined to prepare a 4 nM (or 2 nM) library. Below, we describe methods for measuring DNA mass concentration using a Qubit 4 Fluorometer (Thermo

Fisher Scientific Inc.) and fragment length (bp) using MultiNA (Shimadzu Corporation).

• Quantitation of the DNA library concentration using a Qubit 4 Fluorometer

Necessary consumables

- (1) Qubit™ 1X dsDNA HS Assay Kits (Cat. No. Q33230)

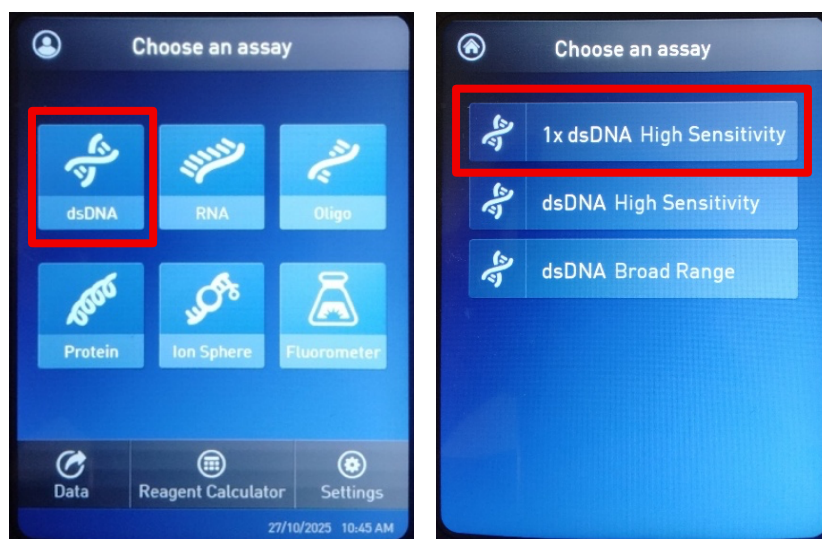
The kit is composed of the following three reagents:

- Qubit™ 1X dsDNA HS Working Solution
- Qubit™ 1X dsDNA HS Standard #1
- Qubit™ 1X dsDNA HS Standard #2

- (2) Qubit™ Assay Tubes (Cat. No. Q32856)

(If previous calibration with the standards is within a month, skip Steps 1, 2, and 7–9)

1. Mix 10 µl Standard #1 and 190 µl Working Solution in Qubit™ Assay Tubes.
 2. Mix 10 µl Standard #2 and 190 µl Working Solution in Qubit™ Assay Tubes.
 3. Mix 1–20 µl DNA sample and 199–180 µl Working Solution in Qubit™ Assay Tubes. The total volume must be 200 µl.
- ※ Do not write the sample name on the side of the tube as this could interfere with the sample read. Write the sample number on the lid of the tube.
4. Vortex the tubes for 2–3 s.
 5. Leave the tubes at room temperature for 2 min.
 6. Turn Qubit on and select “dsDNA” and then “1×dsDNA High Sensitivity.”



7. Select “read standards” for calibration.
8. Insert the tube containing the Standard #1 solution into the sample chamber and press “Read standard”
9. Analyze Standard #2 in accordance with the instructions on the screen.
10. Press “Run samples” and select the sample volume (amount of sample volume used in Step 3) and units.
11. Insert a sample tube into the sample chamber and then press “Read tube.”
12. The screen will display the results.

• Quantitation of DNA length by *MultiNA*

Necessary consumables

- (1) DNA-1000 Reagent Kit* for MultiNA (292-27911-91; Shimadzu Corporation)

The kit is composed of the following two reagents:

- Separation buffer
- Marker solution

* Other reagent kits are available depending on the target DNA fragment length. However, the DNA-1000 Reagent Kit is suitable for DNA library adjustment for the MiSeq system.

- (2) SYBR® Gold Nucleic Acid Gel Stain (S-11494; Invitrogen)
- (3) φX174 DNA/HaeIII Markers (G1761; Promega)
- (4) RA Chip Cleaning Kit (292-35925-91; Shimadzu Corporation)
- (5) Screw cap tube (60.558; Sarstedt)
- (6) Reaction tube, 0.5mL, PP (72.698.200.02; Sarstedt)

Pre-Analyses checks

- Rinse water is replenished.
- Waste container is properly installed and not full.

This section explains the preparation for MultiNA analysis using On-Chip mode.
(Create a project before analysis. See Manual.)

1. Perform “Probe Rinse” to remove air bubbles from the flow path.
Select **[Wash]** and then **[Probe Rinse]** on the **[Instrument]** menu.
2. Perform “Chip Cleaning.”
Transfer 1.8ml* of Chip Cleaning Solution-RA to a screw cap tube (60.558; Sarstedt) and place it in the Chip cleaning solution position. Select **[Chip Cleaning]** and then **[All Chips]** on the **[Instrument]** menu.

* To simultaneously clean all four microchips, 1.8 mL of Cleaning Solution is required.

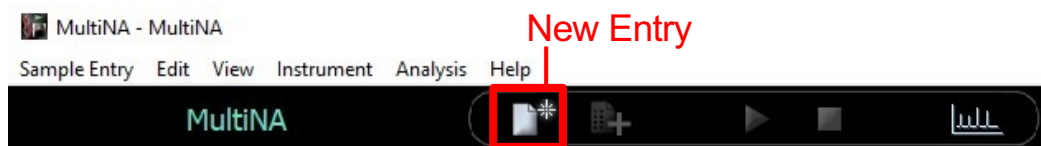
Chip cleaning solution position



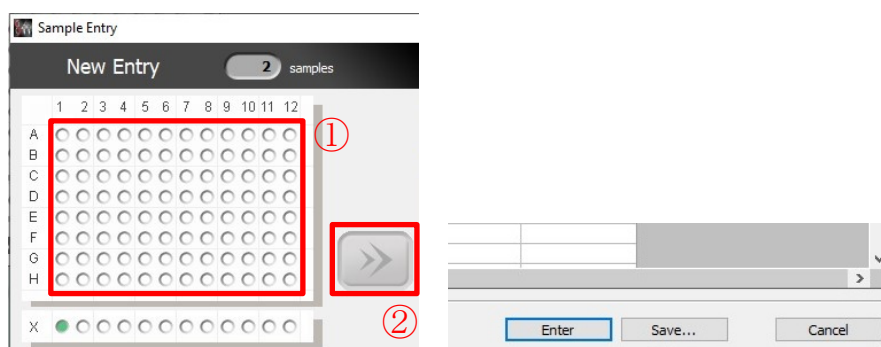
Note: If analysis was performed on the previous day, Chip Washing may be used instead of Chip Cleaning.

Preparation for analysis

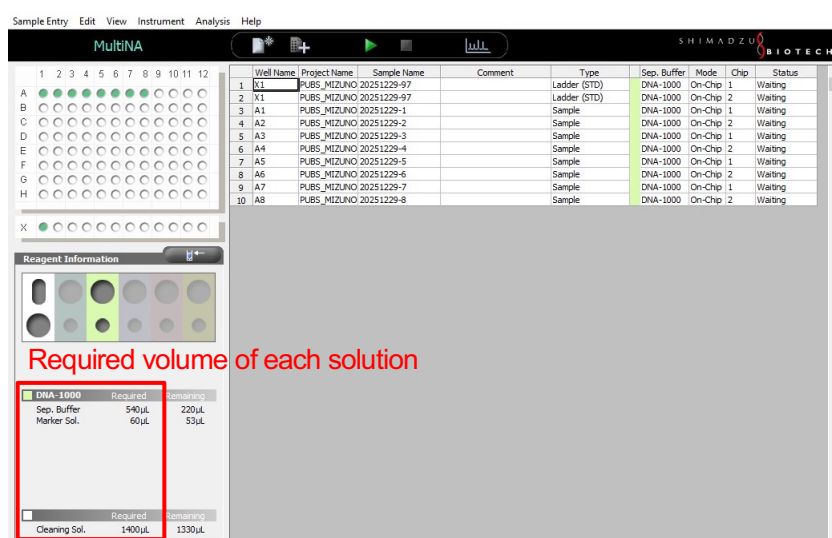
0. Transfer a small amount (<10μl) of SYBR® Gold and dilute 100-fold with TE buffer. Transfer a small amount (<10μl) of φX174 DNA/HaeIII markers and dilute 100-fold with TE buffer. The diluted solutions can be reused. After use, store samples at –20 °C for future analyses.
1. Click “New Entry” and select your project.



2. Select the sample positions in ①, and then click ② and “enter.”



3. The required volumes of each solution are automatically calculated according to the number of your samples. Transfer each solution into the manufacturer-specified tubes.



Seq. Buffer: Mix 100-fold diluted SYBR® Gold (see step 0) and separation buffer* at a 1:99 ratio in a screw-cap tube (60.558; Sarstedt) and vortex to mix.

Maker Sol.: Transfer the required volume of Marker Solution* to a 0.5ml reaction tube (72.698.200.02; Sarstedt).

* Separation Buffer and Marker Solution are included in the DNA-1000 Kit

Cleaning Sol.: Transfer the required volume of Chip Cleaning Solution-RA to a Screw cap tube (60.558; Sarstedt).

4. Transfer samples and ladder* to 8-strip tubes. Place each sample and solution tube in the appropriate position.

Seq. Buffer: ①

Maker Sol.: ②

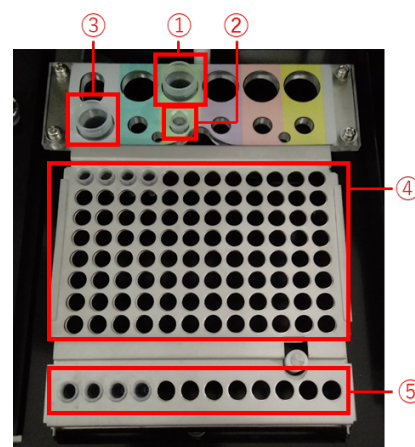
Cleaning Sol.: ③

Samples**: ④

Ladder: ⑤

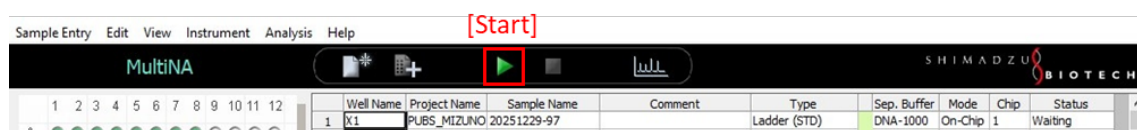
* 100-fold diluted φX174 DNA/HaeIII markers (see step 0). The volume required depends on the number of analyses of the ladder (See Manual).

** When the sample concentration exceeds 50ng/μL, dilute it with TE buffer. When the number of samples is <13, the sample volume required is 9μL.



See the Manual if it is >12.

5. Install the sample holder
Be careful not to bump the sample and ladder tubes when installing the sample holder. Sample spatter caused by an impact may prevent samples and ladder from being suctioned properly.
6. Click **[Start]** on the toolbar. Analysis starts after the instrument status check.



• Preparation of the 4 nM (2 nM) DNA library

You can adjust the 4nM (or 2nM) DNA library with results from Qubit and MultiNA.

1. Open the Excel file “Miseq_ConcCalc.xlsx”* and enter the concentration measured by Qubit in column B. Enter the mean length of the DNA library estimated based on the MultiNA analysis results into column C. Enter the target concentration (normally 4 or 2 nM) in column E and the volume of the DNA library used in column F.

* The Excel file can be downloaded from doi.10.14989/kds618407.

	A	B	C	D	E	F	G
1	Sample	ng/μL	mean size	nM	target (nM)	Template (μl)	TE (μl)
2	rbcL	5.10	490	15.77	4	10	29.4
3	ITS (sample 1)	4.40	460	14.49	4	10	26.2
4	ITS (sample 2)	0.90	460	2.96	2	10	4.8

Using the four input values in columns B, C, E, and F, the implemented functions calculate molar concentration (nM) of your library in column D and the volume of TE buffer required to dilute it to the target concentration in column G.

2. Mix the volume of the DNA library entered in column F with that of TE buffer indicated in column G to achieve the target concentration in column E.
- ※ Basically, the final DNA library is prepared at 4nM. Only when the concentration of the DNA library is lower than 4nM, prepare the final library at 2nM.

• Description of the calculations performed in the Excel file

First, the mass concentration (ng/μl) of the DNA library (Column B) is converted into molar concentration (Column D).

$$\text{Column B (ng/μl)} \times 10^6 \div \text{Column C (bp)} \div 660 = \text{Column D (nM)}$$

※ 660: Average molecular weight of the nucleotide pair

Second, calculate the required volume of TE buffer to adjust the DNA library to 4nM

$$\text{Column D} \times \{ \text{Column F} / (\text{Column F} + \text{Column G}) \} = 4\text{nM}$$

The concentration of the DNA library (Column D) has already been calculated. The volume of the DNA library used (Column F) is determined by you. Therefore, the required volume of TE buffer (column G) can now be calculated using the formula above.

2. MiSeq Run Protocol

H. Sato and T. Mizuno

1. Sample sheet preparation and registration

There are several ways to create and register a sample sheet. One example is provided here. For details, refer to the information provided by Illumina. The sample sheet is a comma-separated values (*.csv) file that stores the information required to set up, perform, and analyze a sequencing run.

- ① Manually create your sample sheet using a text editor (e.g., Excel) based on the example below. Modify the yellow-highlighted sections according to your MiSeq run and save it as a csv file.

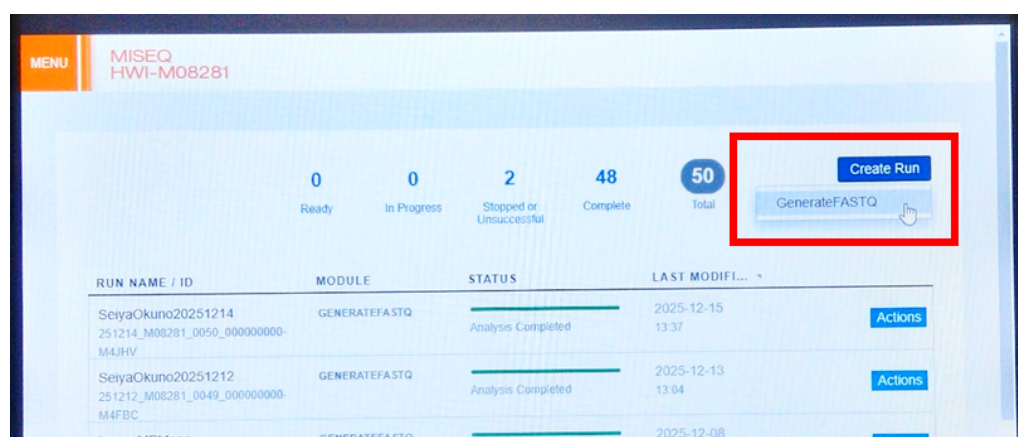
	A	B	C	D	E	F	G	H
1	[Header]							
2	Local Run Manager Analysis	23023						
3	Experiment Name	Hirotohi_Sato						
4	Date	2025/12/30						
5	Module	GenerateFASTQ - 3.0.1						
6	Workflow	GenerateFASTQ						
7	Library Prep Kit	Custom						
8	Index Kit	Custom						
9	Chemistry	Amplicon						
10								
11	[Reads]							
12		251						
13		251						
14								
15	[Settings]							
16								
17	[Data]							
18	Sample_ID	Sample_Name	Description	I7_Index_ID	index	I5_Index_	index2	Sample_Project
19	XPDC-SIM_0015		8 1-A1		GTGTCTCT		GGCCATAT	
20	Kapit-FMU_00313		16 1-A2		TAGGAACG		GTACGATC	
21	NHNP_0017		24 1-A3		ATCCTAGG		ACCTGTTC	
22	BRNP_0073		32 1-A4		ATAAGCGG		GTCACAGT	
23	GNCP_0017		40 1-A5		ACCTCTAG		ATATCCGC	

Annotations:

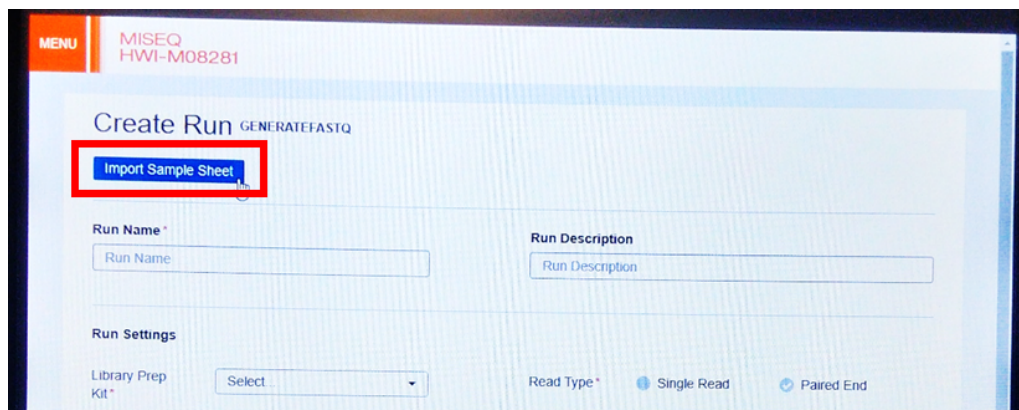
- Name of experimenter (points to B3: Hirotohi_Sato)
- Date of Miseq run (points to B4: 2025/12/30)
- Change the numbers according to your Miseq kit
76 (for 150 cycle)
251 (for 500 cycle) (points to B12 and B13)
- Each sample ID must be unique (points to A19)
- Reverse complements of i7 index sequences (points to E19)
- i5 index sequences (points to G19)

i7 index sequences in the sample sheet must be reverse complements of the actual index sequences.

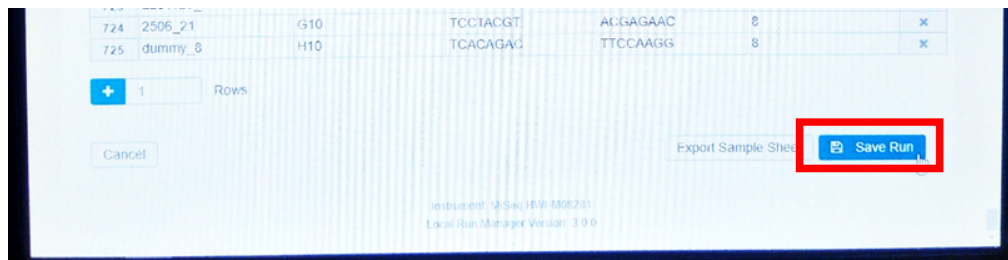
- ② Open Local Run Manager in Miseq, then click “Create Run” and “GenerateFASTQ.”



- ③ Click “Import Sample Sheet” and select your sample sheet.



- ④ Confirm that the imported information is correct. Scroll down to the bottom of the screen and click “Save Run.” An error will occur if any sample ID or index combination is not unique.



- ⑤ Close Local Run Manager.

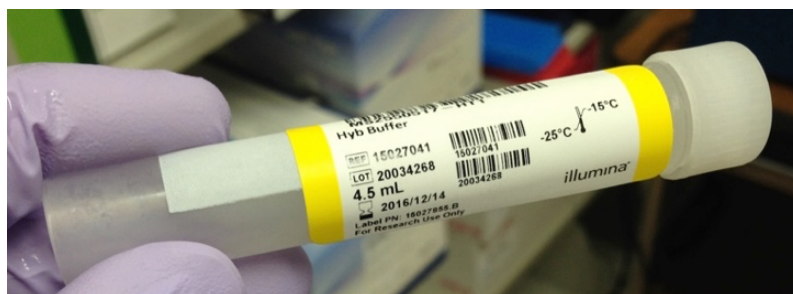
2. Preparation of the MiSeq reagent kit

The MiSeq Reagent Kit consists of frozen and refrigerated reagents. The frozen reagent determines the length of sequencing (number of sequencing cycles). The refrigerated reagent determines the number of sequencing reads (e.g., v2 Nano = 1,000,000 reads and v2 Normal = 15,000,000 reads). You need to choose the reagents according to your aim.

- ① Put the frozen reagent in the refrigerator one day before Miseq run to defrost it. When defrosting on the same day, put the cartridge in water for 30–60 min (please be mindful of the max water line). After defrosting, wipe off water and store the reagent in the refrigerator.



- ② Put HT1 Buffer (↓) in the refrigerator or on ice. The HT1 Buffer is included with the cartridge in the frozen reagents.



3. Adjust 0.2 N NaOH

- ① Remove a tube of 1N NaOH from the freezer and thaw it. (Dispense 200 or 50 μ l of 1N NaOH into tubes and store them in a deep freezer in advance.)
- ② Adjust 0.2 N NaOH as follows:
- | | |
|------------------|---------------------|
| 1N NaOH | 50 (200) μ l |
| H ₂ O | 200 (800) μ l |
| 0.2 N NaOH | 250 (1,000) μ l |
- ③ Confirm that the pH of 0.2 N NaOH is higher than 13 using pH test paper (you can omit this step).

4. Denature and adjust the DNA library

If you start the experiment from the 4 nM DNA library

- ① Add 0.2 N NaOH to the 4 nM DNA library to denature DNA.

4 nM DNA library	5 μ l
0.2 N NaOH	5 μ l
2 nM denatured library	10 μ l

- ② Vortex and spin the tube.
- ③ Incubate the tube for 5 min at room temperature.
- ④ Add 5 μ l of 200mM Tris-HCL to neutralize the NaOH.
- ⑤ Add 985 μ l of HT1 buffer. (You can get 1mL of 20 pM DNA library).
- ⑥ Place the library on ice or in the refrigerator.

If you start the experiment from the 2 nM DNA library

- ① Add 0.2 N NaOH to the 4 nM DNA library to denature the DNA.

2 nM DNA library	10 μ l
0.2 N NaOH	10 μ l
1 nM denatured library	20 μ l

- ② Vortex and spin the tube.
- ③ Incubate the tube for 5 min at room temperature.
- ④ Add 10 μ l of 200mM Tris-HCL to neutralize the NAOH.

- ⑤ Add 970 μl of HT1 buffer. (You can get 1 mL of 20 pM DNA library).
- ⑥ Place the library on ice or in the refrigerator.

5. Denature and adjust PhiX control

If you adjust PhiX from the stock solution (10 nM frozen PhiX)

- ① Defrost the frozen 10 nM PhiX.
- ② Adjust PhiX to 4 nM.

10 nM PhiX	2 μl
H ₂ O (PCR grade)	3 μl
4 nM PhiX	5 μl

- ③ Add 0.2 N NaOH and denature PhiX (you can get 2 nM of denatured PhiX).

4 nM PhiX	5 μl
0.2 N NaOH	5 μl
2 nM denatured PhiX	10 μl

- ④ Vortex and spin the tube. Incubate the tube for 5 min at room temperature.
- ⑤ Add 990 μl of HT1 buffer (You can get 1 mL of 20 pM PhiX).
- ⑥ Place the library on ice or in the refrigerator.

(Note) If you store 20 pM denatured PhiX in the freezer, you can reuse it (but the quality will be decreased).

If you reuse the frozen 20 pM PhiX

- ① Defrost 20 pM PhiX.
- ② Incubate the PhiX at 96°C for 2 min. Cool the tube on ice.

6. Adjust final DNA library (mix DNA library and PhiX)

- ① Mix the DNA library, PhiX, and HT1 buffer.

20 pM PhiX	30 μl
20 pM DNA library	240 μl
Chilled HT1 Buffer	330 μl
9 pM final DNA library	600 μl

(Note) Manufacturer's recommended 10 pM, but we reduce the DNA concentration to avoid overcluster. Generally, please add PhiX to the final library, but this is not obligatory.

- ② Create a hole in the #17 port in the MiSeq cartridge using a 1000- μl tip and discard the tip. Using a new tip, transfer 600 μl of the final library to the #17 port.

7. Sequencing

Please also check Illumina-provided Manual.

- ① Click the "Sequence" button in the MiSeq PC.
- ② If necessary, enter the BaseSpace ID and password (If you use the sample sheet registered in Local Run Manager, skip this step).

- ③ Flow cell cleaning.
Put on a new pair of powder-free gloves and remove the flow cell from the container (The flow cell is included in the refrigerated reagent). Lightly rinse the flow cell with laboratory-grade water until both the glass and plastic cartridge are thoroughly rinsed of excess salts. Using care around the black flow cell port gasket, thoroughly dry the flow cell glass and plastic with a lint-free lens-cleaning tissue. Gently pat dry the area of the gasket and adjacent glass. Ensure that the glass is free of streaks, fingerprints, and lint or tissue fibers. Inspect the flow cell port for obstructions.
(Optional: Clean the flow cell glass with an alcohol wipe. DO NOT USE THE ALCOHOL WIPE ON THE FLOW CELL PORT GASKET. Dry excess alcohol with a lint-free lens-cleaning tissue.)
- ④ Set the Flow Cell on the MiSeq.
- ⑤ Discard the solution in a waste liquid bottle and place a PR2 bottle in the MiSeq (the PR2 bottle is included in the refrigerated reagent).
- ⑥ Place the MiSeq cartridge on the MiSeq (after transferring the DNA library into the #17 port).
- ⑦ Check information on the registered sample sheet and click “Next.”
- ⑧ Perform a pre-run check.
- ⑨ Initiate the run.

8. Post-Run Wash

- ① After confirming the message “Run Completed without Error,” proceed with the Post-Run Wash.
- ② Add 5 ml of Tween20 to a 50ml tube (note that you cannot use a pipette to collect Tween20 because of its high viscosity). Then, dilute Tween20 to 50 ml with MilliQ (you get 10% Tween20).
- ③ Vortex carefully to melt the Tween20.
- ④ Add 25 ml of 10% Tween20 to a 500-ml wash bottle. Dilute to 500ml with MilliQ (you get 0.5% Tween20). You can use the remaining 10% Tween20 for the next run (note that you should use it within one month).
- ⑤ Prepare a new 1.5 ml tube. Mix 30 μ l of sodium hypochlorite solution with 870 μ l of MilliQ (0.2% sodium hypochlorite solution).
- ⑥ Prepare a new 1.5 ml tube. Mix 50 μ l of 0.2% sodium hypochlorite solution with 950 μ l of MilliQ (you get 0.01% sodium hypochlorite solution).
- ⑦ Add 1mL of 0.01% sodium hypochlorite solution to the washing tube. Put the washing tube in port #17 in the wash tray.



- ⑧ Fill all the ports of the wash tray, except for port #17, with 0.5% Tween20.
- ⑨ Remove the used PR2 buffer and waste bottles. Discard liquid in the waste bottle. Put a 500ml bottle with Tween 20 and the waste bottle in MiSeq.
- ⑩ Remove used sequencing cartridge and put the wash tray in MiSeq.
- ⑪ Start the Post-Run Wash.

3. Sequence data processing using the Claident software

H. Sato and T. Mizuno

Installation of the Claident software (Linux / Mac OS)

- Execute the following commands to install the Claident software.

```
mkdir -p ~/workingdirectory
cd ~/workingdirectory
wget https://www.claident.org/installClaident_Debian.sh
sh installClaident_Debian.sh
wget https://www.claident.org/installOptions_Debian.sh
sh installOptions_Debian.sh
wget https://www.claident.org/installDB_Debian.sh
sh installDB_Debian.sh
wget https://www.claident.org/installUCHIMEDB_Debian.sh
sh installUCHIMEDB_Debian.sh
cd ..
rm -r workingdirectory
```

- Execute the following commands to install the bcl2fastq software.

```
sudo apt-get install alien
wget ftp://webdata.webdata@ussd-ftp.illumina.com/Downloads/Software/bcl2fastq/bcl2fastq-1.8.4-Linux-x86_64.rpm
sudo alien -i bcl2fastq-1.8.4-Linux-x86_64.rpm
sudo apt-get install libxml-simple-perl_xsltproc
```

- Edit bcl2fastq as follows:

Change

```
sudo perl -i -npe 's/qwn(ELAND FASTQ FILES PER PROCESSn)/n("ELAND FASTQ FILES PER PROCESS"n)/' /usr/local/lib/bcl2fastq-1.8.4/perl/Casava/Alignment/Config.pm
```

into

```
sudo perl -i -npe 's/qwn(ELAND GENOMEn)/n("ELAND GENOME"n)/' /usr/local/lib/bcl2fastq-1.8.4/perl/Casava/Alignment/Config.pm
```

Conversion of BCL files into fastq files

- Import the run data folder to your computer. This folder is placed in “D:\Illumina\MiseqAnalysis” by default.
- Execute the following commands to convert BCL files (basecall files) into fastq files (sequence files with quality data):

```
cd <run folder>/Data/Intensities
```

Note: Here, you specify the working directory. If the run folder name is “M04195_0021_000000000-D263E”, the command is “cd M04195_0021_000000000-D263E/Data/Intensities”.

```
configureBelToFastq.pl ¥
--fastq-cluster-count 0 ¥
--use-bases-mask Y300n,Y8,Y8,Y300n ¥
--input-dir BaseCalls ¥
--output-dir Output
```

Note: Here, the “use-bases-mask” option specifies the sequence length of the forward read, i7 (reverse) index read, i5 (forward) index read, and reverse read. This command assume that you use “MiSeq Reagent Kit v3(600 cycle)”. If you use “MiSeq Reagent Kit v2(500 cycle)”, you need to change this to “Y250n,Y8,Y8,Y250n”. Specify the directory containing BCL files using the “input-dir” option. You also specify the output directory using the “output-dir” option.

```
cd Output
make -j 18
```

Note: On the first line, you change the working directory. On the second line, you convert BCL files into fastq files. Then, you can obtain four files (compressed fastq files) in “Intensities/Output/ <Project name>/Sample_lane1”.

Demultiplex

Here, you separate the fastq files according to samples and genes. To do this, you need to prepare the i5 and i7 index files, as well as the forward and reverse primer files. The format of these files (fasta format) is as follows:

[Index file]

```
>Index1
CGCTCATT
>Index2
ACGTACGT
```

[Primer file]

```
>ITS1
NNNNNNGGAAGTAAAAGTCGTAACAAGG
>ITS2
NNNNNNGCATCGATGAAGAACGCAGC
```

Note: Save the i5 and i7 index files, as well as the forward and reverse primer files as “index2.fasta,” “index1.fasta,” “forward-primer.fasta,” and “reverse-primer.fasta.” These files should be placed in “Data/Intensities.” Note that sequences of “index2.fasta” need to be reverse-complement sequences similar to the Sample Sheet.

- Execute following commands to perform demultiplexing:

```
cd ..
```

Note: Here, the working directory is changed to “Data/Intensities.”

```

clsplitseq ¥
--runname=ラン ID ¥
--index1file=index1.fasta ¥
--index2file=index2.fasta ¥
--primerfile=forward-primer.fasta ¥
--reverseprimerfile=reverse-primer.fasta ¥
--minqualtag=30 ¥
--numthreads=18 ¥
Output/<Project ID>/Sample_lane1/lane1_NoIndex_L001_R1_001.fastq.gz ¥
Output/<Project ID>/Sample_lane1/lane1_NoIndex_L001_R2_001.fastq.gz ¥
Output/<Project ID>/Sample_lane1/lane1_NoIndex_L001_R3_001.fastq.gz ¥
Output/<Project ID>/Sample_lane1/lane1_NoIndex_L001_R4_001.fastq.gz ¥
Demultiplex

```

Note: You specify the run name by the “runname” option (you can use an arbitrary name). You specify the i7 index, i5 index, forward primer, and reverse primer files by “index1file,” “index2file,” “primerfile,” and “reverseprimerfile”, respectively. You can specify the threshold value for the sequencing quality of index reads using the “minqualtag” option (index reads with lower than Q30 are not used). If you use parallel computing, please provide the number of CPU using the “numthreads” option (in this case, 18 CPUs are used). You need to specify the input files. Input files correspond to four types (R1, R2, R3, and R4) in “Output/<Project ID>/Sample_lane1” (the name of the Project ID differs according to the MiSeq run). You need to specify these files in the order of R1, R2, R3 and R4. Finally, you specify the output directory (“Demultiplex” is used in this case, but you can use an arbitrary name).

Assembling of forward and reverse reads

- Execute the following command to assemble the forward and reverse reads:

```
clconcatpair --mode=OVL --numthreads=18 Demultiplex Concat
```

Note: Here, you can specify the number of CPUs used for the analysis by the “numthreads” option. You need to specify the input directory (same as the output of the “clsplitseq” command) and output directory (“Concat” is used in this case, but you can use an arbitrary name).

Removal of reads with low quality

- Execute the following commands to remove low-quality reads:

```

for f in Concat/*.fastq.gz ¥
do clfilterseq ¥
--output=folder ¥
--append ¥
--minqual=30 ¥
--minqualen=3 ¥
--maxplowqual=0.1 ¥
--minlen=100 ¥
--numthreads=18 ¥
$ f Filter ¥
done

```

Note: Here, you can specify the threshold of sequencing quality by the “minqual” option. If qualities of base calls repeat to be lower than the “minqual” value “minqualen” times, subsequent reads from these bases are removed. If the remaining reads are shorter than “minlen,” these reads are removed. If equal or more

than “maxplowqual” of the remaining reads show qualities lower than “minqual,” these reads are also removed. You can specify the number of CPUs used for the analysis by the “numthreads” option. You specify the input files as “fastq.gz” files in the “Concat” directory (the output directory of the “clconcatpiar” command). You can use an arbitrary name for the output directory (here, this directory is named as “Filter”).

Removal of noisy sequences

- Execute the following commands to remove noisy sequences (potentially incorrect sequences):

```
clcleanseqv ¥
--derepmode= FULLLENGTH ¥
--primarymaxnmismatch=0 ¥
--secondarymaxnmismatch=1 ¥
--pnoisycluster=0.5 ¥
--numthreads=18 ¥
Filter/*.fastq.gz ¥
Clean
```

Note: You can specify the number of CPUs used for the analysis by the “numthreads” option. You specify the input files as “fastq.gz” files in the “Filter” directory (the output directory of the “clfilterseq” command). You can use an arbitrary name for the output directory (here, the name “Clean” is used here).

Clustering of reads into operational taxonomic units (OTUs)

- Execute the following command to cluster reads into OTUs:

```
clclassseqv --minident=0.97 --numthreads=18 Clean/primarycluster.denoised.fasta Cluster
```

Note: Here, you can specify the threshold value for clustering OTUs by the “minident” option. This value needs to be optimized to the focal organisms and genes. You should use 0.97 for the fungal rRNA internal transcribed spacer (ITS) region, whereas you should use 1 for the ribulose-1, 5-bisphosphate carboxylase/oxygenase (*rbcL*) gene in plants. You can specify the number of CPUs used for the analysis by the “numthreads” option. An input file for this command is “primarycluster.denoised.fasta” in the Clean directory (the output directory of the “clcleanseqv” command). You can use an arbitrary name for the output directory (which is named “Cluster” here).

Output of summary table

- Execute the following command to output the summary table where the row and column represent samples and OTUs, respectively:

```
clsumclass --output=Matrix Cluster/clustered.otu.gz summary
```

Note: Here, you specify “clustered.otu.gz” in the “Cluster” directory (the output directory of the “clclassseqv” command) as the input file. You can use an arbitrary name for the output file (named as “summary” here). The output file is tab-separated text.

Removal of chimeric sequences using the Uchime software

- Execute the following command to remove chimeric sequences using the Uchime software:

```
clrunuchime --otufilename=Cluster/clustered.otu.gz Cluster/clustered.fasta Uchime
```

Note: Here, the input file is “clustered.fasta” in the Cluster directory (the output directory of the “clclassseqv” command). You can use an arbitrary name for the output directory (named as Uchime here). Among the output files, you should use “nonchimeras.fasta” for subsequent analysis.

- Execute the following command to create “OTU-list.txt,” in which names of nonchimeric OTUs are specified:

```
grep ">" Uchime/nonchimeras.fasta | sed -e 's/>//g' -e 's/;size=[0-9]*;//g' > Uchime/OTU-list.txt
```

- Execute the following command to create the summary table in which chimeric OTUs are removed:

```
clfiltersum --otulist=Uchime/OTU-list.txt summary summary2
```

Note: Here, the input file is “summary” (the output file of the “clsumclass” command). You can use an arbitrary name for the output file (here, the name “summary2” is used).

Taxonomic assignment by DNA barcoding

- Execute the following commands to perform the taxonomic assignment:

```
clidntseq --blastdb=fungi_ITS_genus --numthreads=18 Uchime/nonchimeras.fasta blast_result
```

Note: Here, you specify the database used for the taxonomic assignment. For example, you should specify “fungi_ITS_genus,” “plant_rbcL_genus,” and “animals_COX1_genus” for fungal ITS, plant rbcL, and animal COX1 sequences, respectively. You can specify the number of CPUs used for the analysis by the “numthreads” option. The input file for this command is “nonchimeras.fasta” in the Uchime directory (the output directory of the “clrunuchime” command). You can use an arbitrary name for the output file (here, the output file is named “blast_result”).

```
classigntax ¥
--blastdb=fungi_ITS_genus ¥
--maxpopposer=0.2 ¥
--minratio=4 ¥
--numthreads=18 ¥
blast_result ¥
blast_summary
```

Note: Here, you need to specify the database same as the “clidntseq” command. You can specify the proportion of opposer sequences (sequences whose taxonomic information contradicts with that of most sequences) by the “maxpopposer” option. You can also specify the ratio of supporter sequences to opposer sequences by the “minratio” option. If you need to perform the taxonomic assignment more strictly, you should not use those options or change the values (i.e., decrease “maxpopposer” and increase “minratio”). The input file for this command is “blast_result” (the output directory of the “clidntseq” command). You can use an arbitrary name for the output file (the name “blast_summary” is used here). The output file is tab-separated text.

query	superking	kingdom	subkingd	phylum	class	subclass	order	suborder	family	subfamily	genus	species	forma
B1__ITS1_	Eukaryota	Fungi	Dikarya										
B1__ITS1_	Eukaryota	Fungi	Dikarya										
BG002__I	Eukaryota	Fungi	Dikarya	Basidiomycota	Agaricomycetes	Agaricomycetes	Agaricales		Hygrophoraceae		Hygrophorus	Hygrophorus capreolarius	
BG005__I	Eukaryota	Fungi	Dikarya	Basidiomycota	Agaricomycetes	Agaricomycetes	Russulales		Russulaceae		Lactarius		
BG006__I	Eukaryota	Fungi	Dikarya	Basidiomycota	Agaricomycetes	Phallomycetes	Gomphales		Gomphaceae				
BG010__I	Eukaryota	Fungi	Dikarya	Basidiomycota	Agaricomycetes	Agaricomycetes	Agaricales		Entolomataceae		Entoloma	Entoloma sarcopum	
BG011__I	Eukaryota	Fungi	Dikarya	Basidiomycota	Agaricomycetes	Agaricomycetes	Agaricales		Lyophyllaceae		Lyophyllum		
BG012__I	Eukaryota	Fungi	Dikarya	Basidiomycota	Agaricomycetes	Agaricomycetes	Agaricales		Hygrophoraceae				
BG013__I	Eukaryota	Fungi	Dikarya	Basidiomycota	Agaricomycetes	Agaricomycetes	Agaricales		Entolomataceae		Clitocella	Clitocella popinalis	
BG016__I	Eukaryota	Fungi	Dikarya	Basidiomycota	Agaricomycetes	Agaricomycetes	Agaricales		Bolbitiaceae		Agrocybe		
BG019__I	Eukaryota	Fungi	Dikarya	Basidiomycota	Agaricomycetes	Agaricomycetes	Agaricales		Agaricaceae		Agaricus		
BG020__I	Eukaryota	Fungi	Dikarya	Basidiomycota	Agaricomycetes	Agaricomycetes	Thelephorales		Thelephoraceae		Sarcodon		
BG021__I	Eukaryota	Fungi	Dikarya	Basidiomycota	Agaricomycetes	Agaricomycetes	Thelephorales		Bankeraceae		Bankera	Bankera fuliginosa	
BG022__I	Eukaryota	Fungi	Dikarya	Basidiomycota	Agaricomycetes	Agaricomycetes	Agaricales		Cortinariaceae		Hebeloma	Hebeloma hiemale	
BG027__I	Eukaryota	Fungi	Dikarya	Basidiomycota	Agaricomycetes	Agaricomycetes	Russulales		Russulaceae		Lactarius		
DM002__I	Eukaryota	Fungi	Dikarya	Basidiomycota	Agaricomycetes	Agaricomycetes	Agaricales		Agaricaceae				
E52__ITS1	Eukaryota	Fungi	Dikarya	Basidiomycota	Agaricomycetes	Agaricomycetes	Russulales		Russulaceae		Russula		
HC001__I	Eukaryota	Fungi	Dikarya	Basidiomycota	Agaricomycetes	Agaricomycetes	Agaricales		Tricholomataceae		Megacollybia		
HC002__I	Eukaryota	Fungi	Dikarya	Basidiomycota	Agaricomycetes	Agaricomycetes	Agaricales		Mycenaceae		Mycena		
HC003__I	Eukaryota	Fungi	Dikarya	Basidiomycota	Agaricomycetes	Phallomycetes	Gomphales		Gomphaceae				
HC005__I	Eukaryota	Fungi	Dikarya	Basidiomycota	Agaricomycetes	Agaricomycetes	Agaricales		Tricholomataceae		Laccaria	Laccaria vinacea	
HC005__I	Eukaryota	Fungi	Dikarya	Basidiomycota	Agaricomycetes	Agaricomycetes	Agaricales		Amanitaceae		Amanita		
HC007__I	Eukaryota	Fungi	Dikarya	Basidiomycota	Agaricomycetes	Agaricomycetes	Agaricales		Amanitaceae		Amanita	Amanita incarnatiformis	
HC008__I	Eukaryota	Fungi	Dikarya	Basidiomycota	Agaricomycetes	Agaricomycetes	Russulales		Russulaceae				
HC019__I	Eukaryota	Fungi	Dikarya	Basidiomycota	Agaricomycetes	Agaricomycetes	Agaricales		Physalacriaceae		Armillaria	Armillaria tabescens	

Fig. 1 An example of output by the “classigntax” command (copy and paste the text file to Excel).

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Edited by U. Shimizu-kaya, S. Yamashita and T. Itioka

(Received 18 February 2026; Accepted 18 March 2026; Date of publication 31 March 2026)