

MANUAL ON DNA BARCODING OF VASCULAR PLANTS IN SOUTHEAST ASIA

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Charles Anthon E. Cadorna, Abigail L. Garrino, Rogel Victor D. Mendoza,
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Foreword

The ASEAN Centre for Biodiversity (ACB) continues to support programmes in the ASEAN Member States (AMS) that best protect the rich but increasingly fragile biodiversity of the region. Taxonomy, the science of identifying species, is at the heart of the ACB's biodiversity conservation initiatives because it allows us to know and understand, and thus better conserve the species around us.

Since 2009, the ACB has developed training programmes to strengthen taxonomy knowledge in ASEAN in collaboration with the Japan-ASEAN Integration Fund, the East and Southeast Asia Biodiversity Information Initiative, and the Biodiversity Center of Japan-Ministry of the Environment. This long-term partnership has enhanced knowledge and skills in taxonomy of more than 600 representatives of the AMS, with some echoing their training in their home countries, and thus adding to the number of ASEAN nationals with expanded taxonomy skills.

Elevating knowledge and skills in taxonomy continues to be a critical element in efforts to reduce global biodiversity loss and is particularly significant in the implementation of the goals of the Convention on Biological Diversity (CBD) and in strengthening anti-wildlife trafficking measures in the Region.

This *Manual on DNA Barcoding of Vascular Plants in Southeast Asia* represents efforts of the ACB and its partners, the Institute of Biology of the University of the Philippines Diliman and Biodiversity Management Bureau of the Department of Environment and Natural Resources, to use DNA barcoding in taxonomy in accordance with guidance from the CBD and the Global Taxonomy Initiative, to reduce the taxonomic impediment particularly in the ASEAN. It is a welcome addition to the growing body of knowledge of taxonomy, and a meaningful contribution of the ACB to conserving ASEAN's natural wealth.

This publication will be an important reference for scientists, researchers, and staff of agencies and organisations not only those involved in biodiversity conservation, but also those involved in wildlife enforcement and prosecution. The ACB expresses its appreciation to project partners and participants in the AMS who contributed to the development of this manual. We look forward to further collaborations with partners in Japan and the AMS to increase knowledge and skills in taxonomy that will give us a better understanding of the biological resources we have in the region, and thus contribute more effectively in addressing biodiversity loss.



Dr Theresa Mundita S. Lim

Executive Director

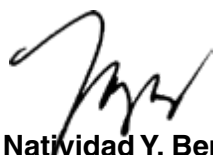
ASEAN Centre for Biodiversity

Foreword

As the focal point for biodiversity conservation in the Philippines, we are pleased to work with the ASEAN Centre for Biodiversity (ACB), Japan-ASEAN Integration Fund (JAIF), and Institute of Biology of the University of the Philippines Diliman on efforts to enhance the taxonomy knowledge and skills of scientists, academics, researchers, and conservation workers in the ASEAN Member States. This project on *Taxonomic Capacity Building on DNA Barcoding of Common Vascular Plants in the Tropics* will surely improve the capacity of our conservation professionals and contribute to global and regional initiatives in reducing biodiversity loss.

Advances in technology allow innovations that improve conservation initiatives and expand collaborations among stakeholders. DNA barcoding, in particular, can be used in a wide range of applications, such as research, wildlife law enforcement, access and benefit-sharing, prevention of the spread of invasive alien species, and ecosystems monitoring and management. This makes training in DNA barcoding significantly useful in our line of work, and beneficial for decision-making and planning for biodiversity conservation and management agencies, scientific and academic institutions, protected areas, and ASEAN Heritage Parks. DNA barcoding can also be used by nature enthusiasts and citizen scientists, which promotes stakeholder participation in biodiversity conservation.

We sincerely thank JAIF for its long-term support to the ACB and its partners in their efforts to strengthen capacity in taxonomy in the ASEAN Member States. We look forward to more collaborations in the future to reduce the taxonomic gaps in ASEAN and contribute to the achievement of the goals of the Convention on Biological Diversity and the Post-2020 Global Biodiversity Framework.



Ms Natividad Y. Bernardino
Director
Biodiversity Management Bureau

Foreword

On April 18–22, 2022, the Institute of Biology, University of the Philippines in Diliman (IB-UPD), together with the ASEAN Centre for Biodiversity, hosted the *Regional Workshop on Taxonomic Capacity Building of Common Vascular Plants in the Tropics*. It was truly an honour and a privilege for our institution to be a part of this workshop in order to share our expertise with our colleagues in the ASEAN towards an integrative approach to taxonomy and conservation biology, particularly with the inclusion of DNA barcoding and bioinformatics. We thank the Japan-ASEAN Integration Fund for making this event possible.

IB-UPD has been at the forefront of DNA barcoding work of native and endemic species in the Philippines. Its DNA Barcoding Laboratory was established in 2008 and has since then forged partnerships with both local and international institutions to populate the Philippine biodiversity database. Our work has been developed in line with the Global Taxonomy Initiative's advocacy on solving the taxonomic impediment. The institute is now in the midst of establishing networks, along with the Department of Environment and Natural Resources, to capacitate regional institutions in the Philippines to protect our biodiversity against illegal wildlife trade. The workshop was part of our efforts to broaden our network to include our colleagues in the ASEAN.

It is our hope that the regional workshop and this *Manual on DNA Barcoding of Vascular Plants in Southeast Asia* will provide the impetus needed to move forward with protecting ASEAN biodiversity through partnerships. We look forward to seeing all our partners very soon and further our collaborations.



Dr Ian Kendrick C. Fontanilla

Director

Institute of Biology

College of Science, University of the Philippines Diliman

Foreword

The Government of Japan, through the Japan-ASEAN Integration Fund (JAIF), has been collaborating with the ASEAN Centre for Biodiversity (ACB) in efforts for capacity building of representatives of the ASEAN Member States (AMS) in taxonomy. Since 2010, JAIF has supported the ACB for the AMS to enhance skills and knowledge in taxonomy, resulting in taxonomy workshops and manuals or field guides on the identification of organisms and plants in Gunung Mulu National Park in Malaysia, and in Doi Inthanon National Park in Thailand. These programmes have successfully trained more than 600 participants from conservation organisations, scientific and academic institutions, protected areas, and ASEAN Heritage Parks in the AMS.

JAIF extended its support to the ACB project *Taxonomic Capacity Building on DNA Barcoding of Common Vascular Plants in the Tropics*, which produced a *Manual on DNA Barcoding of Vascular Plants in Southeast Asia*. The Convention on Biological Diversity (CBD) encouraged Parties of the CBD to use DNA barcoding in taxonomy due to its wider application in various aspects in biodiversity conservation and management. DNA barcoding will be utilised among a greater range of stakeholders, including scientists. Thus, this project provided an opportunity to develop capacity in DNA barcoding among representatives of the AMS, as well as produce two relevant publications – the *Manual on DNA Barcoding of Vascular Plants in Southeast Asia* and the *Field Guide to the Vascular Plants of the Mudspring area, Mount Makiling Forest Reserve, Philippines*. DNA barcodes were obtained for the species included in the field guidebook.

This project and its publications form part of larger efforts to remove the taxonomic impediment in ASEAN, contribute to the achievement of Aichi Biodiversity Targets of the Strategic Plan for Biodiversity 2011–2020, and carry over the goals of the Post-2020 Global Biodiversity Framework.

We are grateful to share this publication with the AMS and other conservation stakeholders and express our appreciation to the ACB as well as our project partners – the Biodiversity Management Bureau, Makiling Center for Mountain Ecosystems, and Institute of Biology of the University of the Philippines Diliman. We look forward to seeing further improvement of related skills and the greater body of knowledge in taxonomy in ASEAN.

Mr Kodama Yoshinori
Minister
Mission of Japan to ASEAN

Preface

The number of species on Earth is estimated to be 10 million – far too many for just a handful of scientists to describe and catalogue. DNA barcoding hopes to alleviate this taxonomic impediment by marrying the best practices of taxonomy with that of DNA technology and bioinformatics. It is our hope that other scientists from all parts of the world adopt this technology and apply it to their respective regions.

With emphasis on vascular plants, this training manual will introduce the concept of DNA barcoding, teach practical skills and processes done in a DNA lab, and impart knowledge on how to analyse DNA data through bioinformatics and other tools. The first part will focus on the conceptual framework on DNA barcoding and plant collection and management. Proper preservation and cataloguing of plant specimens will also be tackled. The second part will dwell on the laboratory component, from DNA extraction to sequence assembly. A discussion on the basic features of the DNA barcoding laboratory will also be highlighted. The third part will emphasise the *in silico* sequence analysis as well as familiarisation of the online database Barcode of Life Data (BOLD) Systems. Actual laboratory data from the training was submitted to BOLD. The fourth part focuses on herbarium management of plant specimen collections.

We hope that by the end of the training, the trainees, at the very least, will be capable of establishing their own DNA Barcoding laboratory and begin populating the database with DNA barcodes of species of tropical plants, particularly in the ASEAN region. This *Manual on DNA Barcoding of Vascular Plants in Southeast Asia* will help support this effort.

We thank the ASEAN Center for Biodiversity, Japan-ASEAN Integration Fund, and the Department of Environment and Natural Resources - Biodiversity Management Bureau, and Makiling Center for Mountain Ecosystems for making the training workshop and publication of this manual possible.

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This training manual was prepared collectively through the submission of up-to-date information of those involved in the activities undertaken by the Japan-ASEAN Integration Fund (JAIF) Project on *Taxonomic Capacity Building on DNA Barcoding of Common Vascular Plants in the Tropics*.

Specifically, thanks are due to the consultants and staff of the ASEAN Centre for Biodiversity (ACB), Dr Edwino S. Fernando, Dr Filiberto A. Pollisco Jr., Sahlee B. Barrer, Nosrat Ravichandran, Rhia C. Galsim, Kyle Esperanza R. Zuleta, Pamela Q. Reblora, Reigna Belle C. Aguja, Walane DC. Aquino, Danielle F. Abrigo, and Rea Anne R. Rañada, for managing the ACB-JAIF project; Atty. Elpidio V. Peria for his discussion on Access and Benefit-Sharing; Dr Ian Kendrick C. Fontanilla, Adrian U. Luczon, and the Institute of Biology-University of the Philippines Diliman (IB-UPD) team for sharing their expertise in DNA barcoding; Ramon Bandong, herbarium assistant and resident expert for plant identification at the Jose Vera Santos Memorial Herbarium at the IB-UPD, for sharing his knowledge with the DNA barcoding workshop participants; and Dr Roberto G. Visco, Leilani A. Castillo, and Nick M. Manlisis of the Makiling Center for Mountain Ecosystems for their assistance in the field work for specimen collections used in the DNA barcoding exercise. Abigail L. Garrino and David R. Ples of IB-UPD collected leaf material and voucher specimens for DNA extraction for the training on DNA barcoding, while Jay E. Matute and Ryan G. Llamas assisted Dr Fernando in collecting leaf material and voucher specimens for the project. The participants of the *Regional Workshop on DNA Barcoding of Vascular Plants of Southeast Asia* held on 18–22 April 2022 in the Philippines also helped refine the contents of the training manual.

The ACB also appreciates Dr Richard Chung Cheng Kong, Forest Research Institute of Malaysia (FRIM); Dr Prachaya Srisanga, Botanical Garden Organization (BGO); Dr Ratchada Pongsattayapipat, BGO; Dr Thamasak Yeemin, Ramkhamhaeng University, Thailand; and Mirasol E. Ocampo, Biodiversity Management Bureau, for critically reviewing an earlier version of this manuscript.

This *Manual on DNA Barcoding of Vascular Plants in Southeast Asia* would not have been possible without the guidance and support of the ASEAN Secretariat, Japan Management Team, and JAIF, and the ACB thanks them for their long-term support of efforts to strengthen taxonomy in ASEAN.



The participants of the *Regional Workshop on DNA Barcoding of Vascular Plants of Southeast Asia* held on 18–22 April 2022 in the Philippines
Photo by Patricia S. Leuterio

Acronyms

ABS	Access and Benefit-Sharing
ACB	ASEAN Centre for Biodiversity
AGE	Agarose Gel Electrophoresis
AHP	ASEAN Heritage Park
AMS	ASEAN Member States
APG	Angiosperm Phylogeny Group
AHTEG	Ad Hoc Technical Expert Group
BGO	Botanical Garden Organization
bp	base pairs
BLAST	Basic Local Alignment Search Tool
BOLD	Barcode of Life Data System
CBD	Convention on Biological Diversity
CITES	Convention on International Trade in Endangered Species in Wild Fauna and Flora
COI	cytochrome c oxidase subunit I
COP	Conference of Parties
dBA	decibels A
ddNTP	dideoxynucleotide triphosphate
dNTP	deoxynucleoside triphosphate
DSI	Digital sequence information
EtBr	Ethidium bromide
EDTA	Ethylenediaminetetraacetic acid
FRIM	Forest Research Institute Malaysia
GBO	Global Biodiversity Outlook
GSPC	Global Strategy for Plant Conservation
GTI	Global Taxonomy Initiative
GTI-DNA-tech	GTI DNA technology training programme
iBOL	International Barcode of Life
ICN	International Code of Nomenclature for algae, fungi, and plants
IPLC	Indigenous people and local community
ISO	International Organization for Standardization
ITS	internal transcribed spacer
ITS2	internal transcribed spacer II
JAIF	Japan-ASEAN Integration Fund
<i>matK</i>	maturase K
MSDS	Material Safety Data Sheet
NCBI	National Center for Biotechnology Information
NBSAPs	National Biodiversity Strategies and Action Plans

NJ	Neighbor-Joining
PCR	Polymerase Chain Reaction
PPE	Personal protective equipment
<i>rbcL</i>	ribulose-1,5-bisphosphate carboxylase
RAP	Regional action plan
RNA	Ribonucleic acid
SBSTTA	Subsidiary Body on Scientific, Technical and Technological Advice
SBI	Subsidiary Body on Implementation
SING	Singapore Botanic Gardens
SSU rRNA	small subunit ribosomal RNA
TAE	Tris-Acetate-EDTA
TBE	Tris-Borate-EDTA
TK	Traditional knowledge
UV	Ultraviolet

Executive Summary

Since 2010, the ASEAN Centre for Biodiversity (ACB) has worked with Japan-ASEAN Integration Fund (JAIF) on various projects to strengthen taxonomy in ASEAN. These include training workshops on the taxonomy of hard corals, freshwater fish, monocot and dicot plants, economically important insects, and bryophytes and pteridophytes. These projects have since trained more than 600 participants from academic institutions, conservation agencies and organisations, protected areas, and ASEAN Heritage Parks (AHPs) in the ASEAN Member States (AMS). These initiatives also generated a number of knowledge products, including field guides, manuals, and workshop reports and proceedings.

The latest of these collaborations is the project on *Taxonomic Capacity Building on DNA Barcoding of Common Vascular Plants in the Tropics*, which produced this *Manual on DNA Barcoding of Vascular Plants in Southeast Asia*. DNA barcoding is a tool for identifying species using DNA sequence information on the designated portion of a specific gene. The 11th Conference of Parties (COP 11) to the Convention on Biological Diversity (CBD) in 2012 first discussed the use of DNA barcoding in taxonomy. COP 13 (Mexico 2016) and COP 14 (Egypt 2018) further reinforced the need for new technologies for capacity building in taxonomy, such as DNA barcoding, to reduce the taxonomic impediment, particularly in the tropics.

The ACB-JAIF project is aligned with the Global Taxonomy Initiative (GTI) Regional Action Plan for Southeast Asia, 2017–2025 (Action 1.2.1.11) to achieve the objective of enhancing the taxonomic capacity of AMS. A major output of the project was the conduct of the DNA barcoding training held in the laboratory of the Institute of Biology, University of the Philippines Diliman, Philippines with participants from the AMS. The training course provided the essential elements of this training manual.

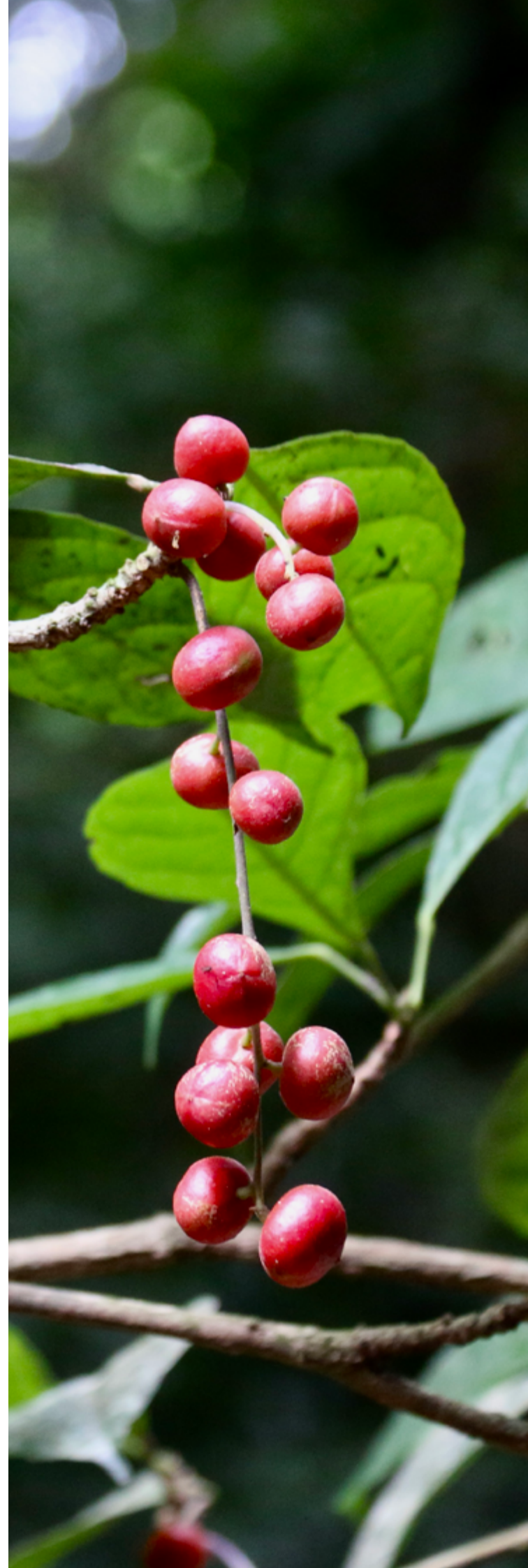
This training course follows the CBD-University of Guelph protocols for DNA barcoding and was modified to include an introduction to taxonomy and a discussion on access and benefit-sharing (ABS). This training manual is made to ensure the consistency of the course's programmes and activities and can be used as a reference for technical staff of the ministries and other stakeholders of the AMS, especially those coming from the academe, botanic gardens, and research institutions conducting species conservation.

This manual provides a background of DNA barcoding in the CBD and the GTI, requirements for the actual training programme, and discussions of the training topics in DNA barcoding. The training content covers four main parts. Part 1 introduces taxonomy, ABS, DNA barcoding, and specimen collection. Part 2 focuses on laboratory exercises where specimens are used for DNA extraction and translated into barcodes. Part 3 discusses sequence analysis and bioinformatics, and how species information is included in global databases. The manual also

discusses herbarium management in Part 4 to provide individuals and organisations with insights on how to establish an herbarium for their specimen collections.

The application of DNA barcoding technology is broad, from biodiversity research to wildlife law enforcement and has the potential to support capacity development for ABS. Learning and sharing this technology and information with AMS can help accelerate the application of DNA technology in species conservation, cross-border trade in wildlife and other live organisms, mitigation of the spread of invasive alien species, and ecosystems monitoring and management.

Baccaurea tetrandra (Baill.) Müll.Arg.
Photo by Edwino S. Fernando



About the Global Taxonomy Initiative

The Conference of the Parties (COP) to the Convention on Biological Diversity (CBD) recognised that the shortage of taxonomic experts and unequal and limited access to taxonomic information (e.g., taxonomic literature, field guides, species identification aids, and others) and collections of natural history specimens hinder many Parties from knowing, managing, and safeguarding their own biodiversity. The recognition of this taxonomic impediment led to the establishment of the Global Taxonomy Initiative (GTI) in 2000. The programme of work of the GTI was adopted in 2002 to advance taxonomy worldwide and provide capacity-building opportunities for countries where the taxonomic impediment exists (Abrahamse et al. 2021).

Since its formation, the GTI has been shaped by voluntary contributions made by the GTI national focal points, scientists, and partners. These led to the (1) assessment of taxonomic needs and capacity at the national or regional levels; (2) establishment, maintenance, and provision of taxonomic information systems, species lists, and databases that increase public knowledge and understanding on biodiversity; (3) capacity building in taxonomy for sectors reliant on taxonomic skills, through forums, networks, workshops, research, and engagement with government bodies; and (4) linkage of taxonomic information to thematic areas and cross-cutting issues under the Convention (Abrahamse et al. 2021).

Since the Strategic Plan for Biodiversity 2011–2020, with its associated Aichi Biodiversity Targets, was adopted in 2010 (decision X/2), the GTI has made steady progress towards Aichi Target 19, which states that “by 2020, knowledge, the science base and technologies relating to biodiversity, its values, functioning, status and trends, and the consequences of its loss, are improved, widely shared, and transferred, and applied” (Abrahamse et al. 2021).

Despite the scientific progress made in the last decade, the fifth edition of the Global Biodiversity Outlook (GBO-5) released in 2020 points to major remaining capacity imbalances and gaps and limited application of biodiversity knowledge to decision-making processes. Comprehensive and up-to-date information about the state of biodiversity, as well as the means to share, access, and use this information, are needed to develop strategies that respond to this threatening decline of global biodiversity and enable transformative change (Abrahamse et al. 2021).

DNA Barcoding and the University of Guelph

DNA barcoding relies upon the assembly of reference sequence libraries that are linked to specimens that have ideally been identified to a species-level, but this can only be achieved for known species. In practice, specimen identification is often impossible because about 90 per cent of all multicellular species await description but every specimen can gain placement in higher taxonomic categories. Moreover, so long as it is deposited in a major collection, its taxonomic placement can be refined through time (Centre for Biodiversity Genomics 2021).

Through the GTI, the Secretariat of the CBD has collaborated with the International Barcode of Life (iBOL) consortium to support Parties in expanding their capacity to discover and understand biodiversity. Aided by iBOL and the University of Guelph, the GTI DNA technology training programme (GTI-DNA-tech) was a five-year effort to address the taxonomic impediment (i.e., lack of scientific experts, taxonomic knowledge, and infrastructure) needed to implement the goals of the Convention (Centre for Biodiversity Genomics 2021).

Over its lifespan, GTI-DNA-tech enhanced technological and scientific capacity among researchers based in developing biodiversity-rich countries where it is most needed. It also raised biodiversity awareness by engaging with citizen scientists to extend capacity development. The data-sharing platform developed by iBOL places biodiversity information in the hands of people who need it to make informed decisions. This access is aiding the protection of biodiversity while also strengthening collaborations among a global network of experts in this field (Centre for Biodiversity Genomics 2021).

ACB-JAIF collaboration on taxonomy in ASEAN

Since 2010, the ASEAN Centre for Biodiversity (ACB) conducted a number of basic to advanced taxonomic capacity building activities in ASEAN with the support of the Japan-ASEAN Integration Fund (JAIF). The training topics focused mostly on using morphological characteristics, including the taxonomy of hard corals, freshwater fish, monocot and dicot plants, economically important insects, and bryophytes and pteridophytes. These programmes successfully trained more than 600 participants from the ASEAN Member States (AMS) since its implementation.

In November 2018, the ACB presented these regional activities in Southeast Asia to the Global Taxonomy Initiatives Forum of the 14th Meeting of the Conference of Parties to the CBD (COP 14) in Sharm El-Sheikh, Egypt. At this CBD forum, DNA barcoding and its application for global taxonomic capacity development was considered and the CBD Secretariat invited the ACB to promote this at a regional level. The GTI Forum revisited the deliverables of the CBD GTI to enhance the use of existing tools and biodiversity informatics and implement effective mechanisms of capacity development by integrating scientific and technical cooperation into the National Biodiversity Strategies and Action Plans (NBSAPs) and their implementation.

The latest of these projects in collaboration with the ASEAN and the JAIF is the project on *Taxonomic Capacity Building on DNA Barcoding of Common Vascular Plants in the Tropics* of which this training manual is one of the major outputs.

Capacity enhancement on DNA barcoding is part of the specific actions identified in the GTI Regional Action Plan (RAP) for Southeast Asia, 2017–2025 (Action 1.2.1.11) to achieve the objective of enhancing the taxonomic capacity of AMS. It was also among the recommendations of the GTI Forum during the COP 14 in Egypt on 16 November 2018 under the theme, *Removing the taxonomic impediment for all citizens*. As such, the project on *Taxonomic Capacity Building on DNA Barcoding of Common Vascular Plants in the Tropics* was proposed by the ACB, approved by the ASEAN, and funded through the JAIF.

Implemented from August 2021 to December 2022, one of the major activities of the project was the conduct of the DNA barcoding training held in the laboratory of the Institute of Biology, University of the Philippines Diliman, in Quezon City, Philippines with participants from the AMS.

This training course followed the CBD-University of Guelph protocols for DNA barcoding. However, as a modification, an additional component of this training course is the application of traditional taxonomy conducted prior to the laboratory exercise. As mentioned earlier, “specimen identification is often impossible because about 90 per cent of all multicellular species await description” and they are not available in the global databases. More often, field personnel do not have instant access to global databases and would surely rely on traditional taxonomy for plant or animal identification. Hence, this component was built in the course so that during the DNA barcoding exercise in the laboratory, the barcodes generated can easily be known as belonging to the identified species. This will be verified further by teaching the participants to use the global databases.

Lessons learned during the conduct of the laboratory exercises are thus incorporated in this training manual so as not to repeat mistakes and encourage uptake of good lessons.

This training manual was developed as an output of this training activity and the project as a whole.

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- Centre for Biodiversity Genomics, University of Guelph. (2021). *The Global Taxonomy Initiative 2020: A Step-by-Step Guide for DNA Barcoding*. Technical Series No. 94. Secretariat of the Convention on Biological Diversity, Montreal, 66p.

DNA barcoding is a tool for identifying species using its DNA sequence information on the designated portion of a specific gene. The use of DNA barcoding in taxonomy was officially introduced at the Eleventh Conference of Parties (COP 11) to the Convention on Biological Diversity (CBD) in 2012. Initiatives from the COP 13 (Mexico 2016) and COP 14 (Egypt 2018) further reinforced the need for capacity building for new technologies related to taxonomy, such as DNA barcoding, to reduce the taxonomic impediments, especially in the tropics.

The application of DNA barcoding technology is broad, from biodiversity research to wildlife law enforcement and has the potential to support capacity development for access and benefit-sharing (ABS). Learning and sharing this technology and information with ASEAN Member States (AMS) can help accelerate the application of DNA technology in conservation, cross-border trade in wildlife and other live organisms, mitigation of the spread of invasive alien species, and ecosystems monitoring and management.

Using bioinformatics in systematics (grouping organisms based on the similarity of DNA sequences) is increasingly being used in biodiversity conservation, both in the field and laboratory, and has become a powerful tool for discovering and observing understudied and species-rich taxa.

Taxonomic knowledge and digital information, which include DNA barcode libraries, are also growing and becoming available to governments and the public. However, the CBD Parties also recognise the need to further enhance tools development and training, which are highly important to allow all sectors to become “bio-literate.” The CBD further acknowledges the need for international collaboration to address the information gap, especially on understudied taxa and regions, including the tropics. Sharing such taxonomic knowledge in an open and free manner could support the development of the Post-2020 Global Biodiversity Framework besides advancing biodiversity science. DNA technologies, such as DNA barcoding, can meet this need for conservation and sustainable use of biodiversity.

Rationale and objectives of the *Taxonomic Capacity Building on DNA Barcoding of Common Vascular Plants in the Tropics* project

DNA analysis technology is available to only a few AMS. However, DNA barcoding, as applied to biodiversity, is a relatively recent technology and can be used especially in curbing illegal wildlife trade aside from enhancing the science of taxonomy. This training manual would help share this technology with all AMS to enhance the taxonomic capacity of the region. The training programme in this document complements the previous traditional training sessions using morphological characteristics, thus expanding taxonomic expertise in the Region.

The main objective of the DNA Barcoding exercise is to enhance the capacity of the AMS in using the DNA-based approach in species identification and discovery. Specifically, the training aimed to: (1) enhance the taxonomic capacity of the staff and researchers from botanic gardens, academic and research institutions, and government units working on species conservation in ASEAN, particularly in applying the standard DNA barcoding in taxonomy as provided in the GTI DNA technology training programme (GTI-DNA-tech) manual, and supplemented by traditional taxonomic procedures; and (2) improve the body of knowledge on the taxonomy of common vascular plants in the tropics, particularly in the ASEAN region.

Purpose of the *Manual on DNA Barcoding of Vascular Plants in Southeast Asia*

This training manual is made to ensure the consistency of the course's programmes and activities as it is applied by the user, particularly the AMS.

The standard GTI-DNA-technical manual developed by the University of Guelph covers protocols in specimen collection management and bioinformatics. On the other hand, this training manual integrates the protocols and procedures prior to “specimen collection management and bioinformatics” so that training skills, processes, and other information necessary to perform the tasks in conducting this course is gathered in one document. This would create significant synergies between the GTI-DNA-tech training, and the traditional training on morphological characteristics, especially for users that do not have access to global databases.

Target of the Training Manual

This manual targets the technical staff of the ministries and other stakeholders of the AMS, especially those coming from the academe, botanic gardens, and research institutions doing species conservation. The DNA information derived from the common indigenous species will fill the information gap in the global databases.

Content of the Manual

This training manual includes discussions on the requirements of a laboratory on DNA barcoding, so that AMS could apply the standard protocols of DNA barcoding in their respective laboratories. The training covers four parts. Part 1 introduces taxonomy, the concept of DNA barcoding, and specimen collection. Part 2 focuses on laboratory exercises where specimens are used for DNA extraction and translated into barcodes. Part 3 discusses sequence analysis and bioinformatics, and how species information is included in global databases. Part 4 focuses on herbarium management of plant specimen collections.

This *Manual on DNA Barcoding on Vascular Plants in Southeast Asia* provides guidance for conservation organisations that aim to conduct similar training programmes for stakeholders in biodiversity conservation. Planning for the training includes choosing the right candidates for training and inviting qualified experts to ensure that knowledge and skills in DNA barcoding are applied and utilised. Feedback and monitoring mechanisms allow the trainers to improve and better prepare for future training events. Organisers also need to ensure that the proper laboratory facilities, equipment, and supplies are available for the training exercise. Certificates of completion should also be prepared particularly for representatives whose organisations require official recognition of their participation in the said training.

Some guidelines are also provided to accommodate both in-person and online participants due to health restrictions imposed by the COVID-19 pandemic.

Pre-Training Requirements

Criteria of Participants

Training on DNA barcoding will benefit technical staff of environment ministries and agencies, as well as representatives from the academe, botanic gardens, and research institutions conducting species conservation work. Staff of protected areas and ASEAN Heritage Parks (AHPs) may use knowledge gained from such training to better understand and catalogue species in protected areas.

Getting information on the participants will help the organisers and resource persons adjust the training programme. This will help satisfy the goals and expectations of both the organisers and participants of the training. See sample participant information in Table 1.

Table 1. Participant Information

Data about the Participant	Data about the Organisation	Participant's Needs and Expectations
Name Contact Information Job Title Gender Age Educational Level Prior training on this subject Current function within the organisation Current work responsibilities	Expected benefits for the organisation	

Criteria of experts

Resource persons in this training programme will require advanced knowledge in these and other relevant subjects:

- Taxonomy
- Forest botany
- Genetics
- Molecular phylogenetics
- Evolutionary biology and systematics
- Population genetics
- Wildlife DNA forensics
- DNA barcoding

The expertise of the resource persons specific to the *Regional Workshop on DNA Barcoding of Vascular Plants in Southeast Asia* and authors of this manual are indicated in page 92.

Logistics

Training on DNA barcoding requires specific facilities, equipment, and supplies. These may not be readily available and should be prepared ahead of the training activity.

A DNA barcoding laboratory requires specialised equipment such as the following: thermal cycler, centrifuge, homogenizer, micropipettes, spectrophotometer or fluorometer, gel electrophoresis set-up, gel imaging and documentation system, vortex mixer, heat block or digital dry bath, autoclave, and microwave. Different materials and supplies are also necessary in different phases of the DNA barcoding exercise, from specimen collection to sequence analysis. All of these must be secured to ensure the success of the DNA barcoding process.

Details of the necessary equipment, materials, and supplies are indicated in each of the processes necessary to conduct DNA barcoding.

Preparing for online participants

Training workshops now have to consider accommodating online participants given the limitations posed by the COVID-19 pandemic. The *Regional Workshop on DNA Barcoding of Vascular Plants in Southeast Asia* was specifically designed for both online and face-to-face participants from the ASEAN Member States (AMS) since some countries still faced travel restrictions. The pandemic also limited the number of participants in the laboratory exercises.

A complete synchronous participation of online and face-to-face participants, however, is not advisable as the workshop covers both lecture presentations and laboratory work. All participants, online and face-to-face, attended the lecture presentations at the same time. For all the laboratory processes, a video was prepared for online participants to save time,

so that they could follow and understand all the steps in the laboratory without having to wait for results or go through the actual time-consuming processes. A livestream of the laboratory work was also not possible given potential connectivity issues as well as the limited number of participants allowed in the laboratory.

In terms of equipment for online participants, organisers need a camera, tripod, laptop with webcam, and stable internet connection. A redundant or backup internet connection is also advised in case there are problems with the primary internet service providers.

The organising team should include a moderator for the online discussions and a competent information technology expert to ensure the availability of all presentations, support the resource persons, guarantee connection with the online participants, and troubleshoot all audio, video, and connectivity problems.

For future similar workshops, it may be more advisable to hold a full face-to-face workshop with laboratory work, and then hold a separate modified workshop, using the video for the laboratory work, for online participants.

Monitoring and evaluation

Training and other capacity building activities require feedback mechanisms where participants can react to various aspects of the training event, such as venue, facilities, logistical arrangements, and accommodations. This allows organisers to review and improve arrangements and enhance the learning environment for future training activities.

Participants should also be able to react to the presentations, method of instruction, and the speakers themselves. This is particularly relevant as participants come from different countries and may have different levels of expertise in relation to the training subjects. Evaluation from the participants provides an opportunity for speakers to better tailor their presentations and teaching methods to an audience with a wide range of skills and experiences.

Certificates of Attendance and Completion

Participation in training programmes, conferences, and seminars add to the skills and knowledge of conservation practitioners and contribute to the achievement of biodiversity conservation goals. Certificates of Attendance or Completion present proof of participation and enhanced capability of the participant to discuss or undertake initiatives relevant to the training received and add to credentials in the event of promotions. Such certificates may also be required to facilitate release of funds in relation to travel and other costs relevant to the training.

Training Structure and Content

The training programme on DNA barcoding covers four main parts. Part 1 introduces traditional taxonomy and DNA barcoding and specimen collection; Part 2 discusses laboratory processes involved in DNA barcoding; and Part 3 focuses on sequence analyses. Part 4 on Herbarium Management discusses the significance, functions, operations, and maintenance of herbaria, which may be of interest to individuals and organisations in the AMS that may want to start organising their collections into an herbarium.

Part 1: Introduction to Taxonomy and DNA Barcoding

The Convention on Biological Diversity, Global Taxonomy Initiative, and DNA Barcoding (0.5 hour)

The discussion provides an introduction to taxonomy, the Convention on Biological Diversity (CBD) and the Global Taxonomy Initiative. It also highlights the history of taxonomy, development of DNA barcoding, as well as the challenges and limitations of DNA barcoding.

Access and Benefit-Sharing Issues in DNA Barcoding (0.5 hour)

The CBD, Nagoya Protocol on Access and Benefit-Sharing (ABS), and national ABS regulations provide guidance in the AMS on access to and use of genetic resources. This is significant as specimen collection is a crucial element in taxonomy. The use of barcodes and other digital sequence information is also an ongoing discussion among Parties to the CBD and may consequently impact DNA barcoding protocols in the future.

Considerations in Permits to Collect Specimens (0.25 hour)

Obtaining permits for specimen collection may involve different government agencies and organisations depending on the protocols of the collection site and the regulations of the AMS. These and other considerations may impact the project timetable and should be considered in the research process. Permit collection may also be discussed in relation to ABS issues.

Collecting plant material for DNA barcoding and Herbarium Voucher Preparation (1 hour)

The session discusses general specimen collection and processes specific to DNA voucher collection, and herbarium voucher collection. Plant collection is the first step in the preparation of herbarium voucher specimens, which provide information on the composition and life of plants in specific sites and their interaction with the natural environment. The genetic material used for DNA barcoding comes from plant material (typically young/fresh leaves) collected in the field, here referred to as “DNA vouchers”. In addition to DNA vouchers that are directly involved in extraction and sequencing, separate “herbarium vouchers” should also be collected as proof of the collected plant’s identity.

Supplies and equipment

- Binoculars
- Pruning shears/pole cutter
- Latex gloves
- Parchment paper
- Crochet threads
- Hole puncher
- Field notebook
- Pencil, eraser, sharpener
- GPS
- Camera
- Any plain flat object to use as a background (e.g. illustration board, folder)
- Teabags or small paper envelopes/ziplock bags
- Silica gel
- A container such as a jar or a box that can fit several tea bags/envelopes
- industrial plastic bags
- Old newspapers
- 70% alcohol
- Straw ball/rope

Drying, Mounting, and Filing of Herbarium Vouchers (0.5 hour)

Herbarium vouchers are used for the identification, classification, and preservation of many different plant species and are crucial to the correct identification of a plant's genus and specific epithet. Proper handling of specimens includes drying, mounting, and filing inside the herbarium storage cabinets.

Supplies and equipment

- Old newspaper
- Wooden herbarium press
- Cardboard
- Corrugated iron sheets
- Rope or binding straps
- Specimen dryer
- Herbarium mounting sheets
- Wood glue or gum tape
- Brush and forceps
- Herbarium label
- Bristol folders

Imaging and Photography (0.25 hour)

Specimen photography allows researchers and other interested parties to view herbarium specimens remotely.

Part 2: Laboratory Processes and Activities

Laboratory Health and Safety (0.5 hour)

Safety protocols are necessary to prevent the contamination of samples, which are particularly important for DNA barcoding. These procedures will also ensure the safety of personnel from biological, physical, and chemical hazards that may be present in the laboratory.

Common Equipment in a Plant DNA Barcoding Lab (0.5 hour)

Specific workspaces and basic equipment are needed to conduct genomic DNA extraction, assessment of DNA quality, polymerase chain reaction (PCR) amplification, and verification of amplification success by agarose gel electrophoresis.

Total Genomic DNA Extraction from Plant Samples (1 hour)

The session describes the different steps in DNA extraction. Extracted DNA is used as a template for DNA amplification. The DNA extraction protocol used is adopted from a commercial DNA extraction kit.

Supplies and equipment

- Analytical balance
- Microcentrifuge tubes (2.0 ml and 1.5 ml)
- Tube racks
- Sterile tips
- Pipettors
- Dry bath
- Microcentrifuge
- Homogenizer
- Steel homogenizer beads
- Plant tissue (e.g., leaves)
- Qiagen® DNeasy Plant DNA Mini Kit
- Crushed ice

Polymerase Chain Reaction Amplification (3 hours)

PCR Amplification utilises the enzyme DNA polymerase to replicate a region of interest in the genome of an organism. The procedure amplifies the DNA region of interest, such as maturase K (*matK*) for plants and ribulose biphosphate carboxylase large chain (*rbcL*) using specific primers that flank the region of interest.

Supplies and equipment

- Sterile 0.2 ml PCR tubes
- Tube racks
- Sterile tips
- Pipettors
- Centrifuge
- PCR machine
- DNA sample
- Ultra-pure water
- PCR buffer
- Primers
- Taq polymerase

Agarose Gel Electrophoresis (1 hour)

The session demonstrates the use of Agarose Gel Electrophoresis (AGE), which involves the separation of biomolecules (e.g., proteins, DNA, RNA) by their charge and size/length. AGE will be used to visualise the PCR products and estimate their fragment length.

Supplies and equipment

- Agarose (Molecular Biology Grade)
- 0.5x/1.0x Tris-Borate-EDTA (TBE) Buffer / 1.0x Tris-Acetate-EDTA (TAE) Buffer
- Nucleic acid stain
- Gel Loading Dye
- 100 bp molecular ladder
- Electrophoresis Set-up
- Gel Casting Materials
- Pipettors

Gel Extraction (1 hour)

The second purpose of the AGE is to separate the desired PCR product if multiple bands appear on the gel. This ensures that the PCR product to be sequenced contains only the amplified fragments of the desired marker and all of them are of the same fragment size. The protocol for gel extraction is adopted from a commercial gel extraction kit.

Supplies and equipment

- Gel solubilisation buffer
- Wash buffer (Absolute ethanol added)
- Elution buffer
- Spin columns
- Collection tubes
- 2.0 ml tubes
- 1.5 ml tubes
- Microcentrifuge
- Pipettors
- Analytical Balance
- Dry bath

DNA Sequencing (0.5 hour)

The session goes through the general processes and concepts in the Sanger sequencing method or chain-termination method developed by Frederick Sanger and colleagues in 1977.

Sequence Assembly (0.5 hour)

The raw DNA sequences must first be assembled prior to sequence analyses. Processing of sequences is necessary to check for ambiguous nucleotides or for gaps. The forward and reverse strands that were amplified through PCR will be used to counter check each other and to build a consensus sequence. The sequence assembly process follows the STADEN package version 1.5.3.

Sequence Alignment (0.5 hour)

The session discusses the procedure for reading processed sequences using DNA sequence analysis software such as BioEdit version 7.0.9 alignment editor.

Part 3. Sequence Analysis

Registering New DNA Barcodes to a Database (1 hour)

The module demonstrates how to upload generated DNA barcode sequences to the Barcode of Life Data System (BOLD), which is an online repository for DNA barcodes of animals, fungi, and plants. Users of this platform can create projects and datasets that can be subjected to several bioinformatic analyses built in.

Bioinformatics tools for species identification (1 hour)

The session provides an orientation on the use of Bioinformatics tools in identifying species. The BOLD identification engine and the Basic Local Alignment Search Tool (BLAST) will be used in the analysis.

Phylogenetic Tree Construction (1 hour)

Using the software MEGA version XI (<http://www.megasoftware.net/>), the module shows how to construct a phylogenetic tree using the Neighbor Joining (NJ) method. Phylogenetic trees are graphical representations of the evolutionary relationships among taxa derived from morphological or molecular datasets. This method identifies the shortest tree by sequentially finding neighbors that minimise the total length of the tree.

Part 4: Herbarium Management (1 hour)

The module discusses the purpose, importance, types, and functions of an herbarium. The discussion will also provide insights into building, using, operating, and maintaining an herbarium, including how to store and access specimens.

Anaxagorea luzonensis A.Gray
Photo by Abigail L. Garrino





PART 1

Introduction to
Taxonomy and
DNA Barcoding

The Convention on Biological Diversity, Global Taxonomy Initiative, and DNA Barcoding

Edwino S. Fernando

The Convention on Biological Diversity (CBD) is a treaty among the vast majority of the world's governments established in June 1992 following the Earth Summit in Rio de Janeiro. Its overall objective is to develop national strategies for the conservation and sustainable use of biological diversity. It sets out commitments for maintaining the world's biodiversity and associated ecological functions. It is a political and legal agreement and identifies priorities for action and puts in place the globally agreed policies to support such actions.

The three main goals of the CBD are:

1. Conservation of biological diversity
2. Sustainable use of its components
3. The fair and equitable sharing of the benefits from the use of genetic resources

The conservation of biodiversity is closely linked with the provision of ecosystem services to support sustainable development. The CBD recognises that its implementation would require a clear understanding of what biodiversity is – scope, components, interactions, functioning – so that it can be sustainably used and conserved.

Taxonomy is the science by which the components of biodiversity are studied, described, identified, named, classified, and understood. Taxonomy delivers the tools to recognise the diversity of life (e.g. identification keys, field guides, databases with taxonomic information, DNA barcodes, and others). Taxonomy, therefore, provides the basic knowledge underpinning biodiversity management and implementation of the CBD.

Plant taxonomists construct the reference system and knowledge base of plant biodiversity that covers:

1. Plant identification – Using characters to recognise and identify plants.
2. Species description – How is a species described?
3. Nomenclature – What should plants be correctly called? How are these names determined?
4. Phylogeny and classification – What are their closest relatives? Are there any other plants likely to have similar properties?
5. Geographic distribution – Where do they grow?
6. Ecology – What kinds of habitat do they prefer?
7. Economic botany – What are their useful properties?

The rules of nomenclature provide that a particular plant anywhere in the world can have one and only one correct and accepted scientific name. The scientific naming of all plants (including organisms traditionally treated as algae or fungi), is governed by a set of rules and recommendations that is called the International Code of Nomenclature for algae, fungi, and plants (ICN). This may be altered or revised at an International Botanical Congress that is held every six years. Only a precise scientific nomenclature associated with the plant collections and DNA barcodes will enable access to further relevant literature and databases.

The Global Taxonomy Initiative

Taxonomic knowledge is far from complete. In the past 250 years of research, taxonomists have named just about 1.78 million species of animals, plants, and microorganisms. However, the total number of species is unknown and probably between 5 and 30 million. In Southeast Asia, while new species of plants are described every year, there is still a large extent of undiscovered species in the region.

Incomplete knowledge of taxa and the general lack of taxonomists have been described by the CBD as a 'taxonomic impediment.' The impediment is most acute in developing countries in the tropics, which contain most of the world's biodiversity, yet produce far fewer taxonomists than developed countries (Geeta et al. 2004). Most taxonomists work in developed, industrialised countries, which typically have less diverse flora than in more tropical developing countries.

Herbaria in industrialised countries also hold most specimens (nearly 70 per cent of 390 million) from the developing countries of the tropics, as well as associated taxonomic information.

The impediment, however, also refers to taxonomy itself. Challenges in taxonomy include the following:

1. Insufficient funding for basic research, such as botanical explorations, herbarium research, and DNA barcoding (Ebach et al. 2011).
2. The regulatory maze associated with collecting biological specimens (Ebach et al. 2011).
3. The low citation rates of taxonomic papers (or low impact factors of most taxonomic journals). Many taxonomists are now promoted, evaluated, compensated, employed, and given or not given tenure on the strength of formulas in which journal impact factors, or citation rates of papers, or numbers of research projects are the variables (Korf 2005).
4. The scattered nature of taxonomic information. The two main products of contemporary taxonomy are (a) hypothesis of species boundaries and (b) hypothesis of the phylogenetic relationships among species. How is the taxonomy of a particular group of plants organised today? Original descriptions of organisms and hypotheses about species identities are scattered in the scientific literature that may span over 250 years and may even be written in various languages (Godfray et al. 2007).

The CBD thus established the Global Taxonomy Initiative (GTI) to remove or reduce the taxonomic impediment, specifically: (1) knowledge gaps in the taxonomic system; (2) shortage of trained taxonomists and curators; and (3) the impact of these deficiencies has on the ability to conserve, use, and share the benefits from biodiversity.

Taxonomy and DNA Barcoding

From the 15th to the 16th centuries, taxonomy benefitted from and improved through the Great Navigations, the invention of the printing press, creation of botanic gardens, and use of the drying technique to preserve plant specimens. Traditional practices in taxonomy have since been largely characterised by collecting, matching, describing, and then publishing discovered species.

Morphology, or the study of the form of living organisms, is the traditional character used in plant taxonomy. Over time, however, new instruments, tools, methods, sources of evidence, and increasing reliance on technology has changed the science of taxonomy. Changes have also come about due to increasing pressure from consumers such as ecologists, conservationists, customs officers, and others.

From the early 1990s, taxonomy entered a new phase based on DNA and information technology. DNA barcodes commenced in the early 2000s and modernised taxonomic practices towards 'taxonomy at the click of a mouse' (Bisby et al. 2002), thus organising, updating, and streamlining the process of bringing taxonomic information into every desktop (or cellphone) with increased speed and accuracy. Large herbaria of the world are now accessible online. Scientists and researchers can now publish new species in online journals (electronic publication) and it is no longer strictly necessary to print journals. Many journals are now published online although few remain 'open access' to readers.

The goal of the DNA barcode initiative is to accelerate the pace of species discovery (Hebert et al. 2003; Savolainen et al. 2005). A DNA barcode constitutes one or more short gene sequences (<1000 base pairs [bp]) taken from a standardised portion of the genome and used to identify species. It also refers to any DNA sequence used for identification at any taxonomic level (Kress 2017; Kress et al. 2015). DNA barcodes function as molecular identifiers for each species, similar to the black and white barcodes used in the retail industry, or the more recent QR codes (Liu et al. 2012; Gogoi et al. 2020; Yu et al. 2017). When a barcode sequence is retrieved from an unknown sample, an algorithm is used to compare it to a reference database containing barcodes from identified museum or herbarium specimens, thus allowing its identification.

From a practical point of view, a key strength of a DNA barcode is that it can be performed on any life stage of the plant – even some that bear no or only few morphological characters, such as seeds or seedlings, and almost any type of material, such as leaves, cambium, bark, dry wood, and roots (Asif & Canon 2005; Rachmayanti et al. 2009; Novaes et al. 2009; Kesanakurti et al. 2011; Yu et al. 2017). DNA barcoding can have forensic applications in that it requires only a small tissue sample from the plant in need of identification and can be used in cases where the sample is degraded, has been processed, or when it consists of only non-characteristic parts of the plant (Rydberg 2010; Ferri et al. 2015; Yu et al. 2017).

The goal of DNA barcoding is to find one or a few regions of DNA that will distinguish among the majority of the world's species and produce a large-scale DNA reference library of life of all species in Earth. However, the rate-limiting step in building a high-quality reference library of DNA sequences will be the collection and assembly of well-identified sample specimen sets (voucher specimens) amenable for DNA sequence analysis (Funk et al. 2005; Hollingsworth 2011; Eisenman et al. 2012; Culley 2013).

Standardisation, minimalism, and scalability are the core principles of DNA barcoding, which should be (Cowan et al. 2006):

1. Short enough to sequence in one piece (~700 bp or less)
2. Technically simple to sequence (e.g. without any long repeat regions that challenge the accuracy of DNA polymerases)
3. Length-conserved to mitigate alignment problems
4. Recoverable from herbarium samples and other degraded DNA samples such as alcohol-preserved tissue, wood, fruits, and others
5. Universal, and can be used for all plants

For plants, the ribulose-1,5-bisphosphate carboxylase (*rbcL*) and maturase K (*matK*) genes in the chloroplast genome are the standard markers for the DNA barcoding approach. Additional markers for plants are the nuclear internal transcribed spacer (ITS), and the non-coding intergenic spacer *trnH-psbA* in the chloroplast genome and several other genes.

Limitations of DNA Barcoding

There are potential strengths and weaknesses of source tissue for developing DNA barcode reference libraries for plants. For example, fresh tissue samples of leaves and flowers would typically yield excellent DNA quality and for any loci; herbarium specimens good DNA quality and for many loci; and wood specimens fair DNA quality and for fewer loci (sapwood) and poor DNA quality and for fewest loci (heartwood) (Jiao et al. 2018). Also, sometimes a combination of two or more barcodes would yield the best discrimination for a number of congeneric species or other barcode combinations to distinguish two anatomically very similar species.

Some specimens may not yield barcodes. Generally, universal primers for polymerase chain reaction amplification of specific segments of the DNA is successful for most species. However, it is reasonable to expect that some species will not yield any PCR product for various reasons. One reason is the non-specificity of the universal primers to the primer-binding site in the genome of the species, which would lead to the failure of the primers to bind to the DNA. Another possible explanation is the presence of specific plant metabolites for specific individuals that may inhibit the PCR process. Some interventions include using a different organ of the plant as source DNA or designing a different set of primers that may anneal to the binding sites in the DNA.

There are also limitations to submitted barcodes, as 74 per cent of cytochrome c oxidase subunit I (COI) barcodes submitted are not identified to the species level (Kwong et al. 2012). For all vertebrates, 89 per cent do not have a voucher reference. There is still a huge percentage of

vertebrate genomes without a voucher reference, specifically mammals (97 per cent), birds (85 per cent), reptiles (88 per cent), amphibians (87 per cent), and fishes (86 per cent) (Buckner et al. 2021).

Plant species DNA barcodes must be vouchered by high-quality extant and digitised reference specimens of the actual samples that were sequenced (Funk et al. 2005; Hollingsworth 2011; Eisenman et al. 2012; Culley 2013).

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Access and Benefit-Sharing Issues in DNA Barcoding

Atty. Elpidio V. Peria

The Convention on Biological Diversity (CBD) sets out the general principles on access and benefit-sharing as the way in which genetic resources may be accessed, and how the benefits that result from their use are shared between users, or the people or countries using the resources, and providers, or the people or countries that provide them. Access and benefit-sharing (ABS) is particularly significant as genetic resources are a key source of taxonomic information and research, which are crucial to effective environmental conservation.

Most countries, including the ASEAN Member States (AMS), have their own ABS regulations that must be complied with as indicated in Art. 15 of the CBD, which states, “the authority to determine access to genetic resources with the national governments and is subject to national legislation.” Guidance on ABS also stems from the Nagoya Protocol on Access and Benefit-sharing, the landmark agreement in the international governance of biodiversity that is particularly relevant to commercial and non-commercial sectors involved in the use and exchange of genetic resources. The Nagoya Protocol applies to (1) genetic resources within the scope of Art. 15 of the CBD and (2) traditional knowledge associated with genetic resources within the scope of the CBD and to benefits arising from the utilisation of such knowledge.

ABS and taxonomy

In relation to ABS and taxonomy, some relevant questions for scientists and researchers include the following:

1. Is my research covered by ABS regulations in the countries of research?
2. How do I comply?
3. How will I know if I do not have to go through those ABS regulations?
4. What happens if I proceed without going through ABS regulations and I found out later I am covered?

ABS brings various challenges, such as the following:

ABS impedes research. This is usually heard from researchers who are unfamiliar with government bureaucracy. In some cases, researchers discontinue their projects, or stop dealing with the relevant ABS regulatory agency. There is also some concern that rather than dealing with the proper regulatory bodies, researchers may find other ways and work with other partners to continue their research projects.

ABS adds layers of bureaucracy. AMS has designated people within the focal agencies to deal with ABS but there are challenges in the interaction among the focal agencies, local government bodies, and other relevant stakeholders including with indigenous peoples and local communities (IPLCs).

ABS adds uncertainties. The ABS agreement between the researcher and the government adds to the uncertainty of what will happen next as the agreement lays out in clear terms the obligations and responsibilities of the Parties to each other.

ABS becomes complicated with the issue of Traditional Knowledge (TK) and IPLCs. Each AMS will have different regulations in relation to ABS, TK, and IPLCs. With the UN Declaration on the Rights of Indigenous Peoples in place since 2007, and the Nagoya Protocol, an AMS will provide its own guidance on establishing measures on how to treat TK and IPLC concerns in ABS settings.

Lack of awareness of the importance of ABS. Access and benefit-sharing regulations are particularly important as they provide the necessary corrective measures to the historical injustice on the utilisation of natural resources from the era of colonisation. This provides the rationale for Art. 15 of the CBD and the establishment of the Nagoya Protocol.

Taxonomic research is usually exempted from ABS regulations, but this should be confirmed with the different ABS regulations in the AMS.

ABS in ASEAN

As seen on the *Scope of the ABS Practice Manual* (p.23), there are many identified activities relating to scientific research that are not covered by the regulations of the AMS that provided information to the ABS Practice Manual. As such, there is a wide scope for researchers on how to package their research projects when applying for permits so that they may not be required to undergo the existing ABS regulation for that particular AMS. However, they may be asked for other reporting requirements as may be specified by these same rules.

As of 17 April 2022, seven AMS are Parties to the Nagoya Protocol. Brunei Darussalam, Singapore, and Thailand have their specific ABS regulations. There is thus a need for a comprehensive document that gives an overview of ABS regulations and arranged in a thematic manner. As a result, the ASEAN Centre for Biodiversity, through the ASEAN-India Cooperation entitled *Capacity Building towards implementing the Nagoya Protocol on Access and Benefit Sharing, the City Biodiversity Index and Strategic Plan on Biodiversity*, produced the *ABS Practice Manual, The Manual of Practice for Access and Benefit-Sharing Approvals and Applications*.

The ABS Practice Manual was developed to:

1. Compile specific practices in the implementation of specific ABS provisions
2. Guide ABS regulators in ASEAN on how other AMS are dealing with similar issues
3. Promote group learning and collaboration and facilitate exchange of information on ABS practices
4. Tighten ABS practice and clarify areas in law and administrative procedure

Scope of the ABS Practice Manual

A. What are covered by the ABS regulations

1. Taking or collection of biological resources, genetic resources, or associated traditional knowledge
2. Research for commercial gain
3. Bioprospecting
4. Others

B. What are NOT covered by the ABS regulations

1. Scientific research on wildlife
2. Research projects involving taxonomic or ecological field studies
3. Non-commercial research
4. Research outside the stated scope of the regulations
5. Collaborative research projects
6. Taxonomic surveys
7. Buying of biological specimens in the market or through peddlers
8. BioTrade
9. Harvesting from plantations of domesticated species
10. Fishing for commerce, recreation or game
11. Taking of animals or plants for food
12. Taking biological resource that has been cultivated or tended for any purpose other than for research and development

13. Taking of natural produce for any purpose other than for research and development
14. Collecting plant reproductive material for propagation
15. Carrying out commercial forestry
16. Use of exchange of biological resource in the exercise of traditional and customary practice of IPLCs
17. Taking of biological resource that is a living modified organism, or a plant variety
18. Access to a biological resource exempted via provision of the law
19. Traditional use
20. Subsistence consumption
21. Existing procedures of collections and transport of wild species
22. Local people and communities practicing indigenous medicine

C. Other exclusions

1. Uses under the International Treaty on Plant Genetic Resources for Food and Agriculture (ITPGRFA)
2. Uses under the Pandemic Influenza Preparedness (PIP) Framework of the World Health Organization



Processing of specimens in the field.

Photo by Belle Aguja

DNA Barcoding and ABS Regulations

DNA barcoding may bypass ABS regulations when a global DNA database becomes operational and the full range of markers of all species is already identified. However, ABS regulations will remain necessary for the collection and extraction of samples in the field.

The Digital Sequence Information (DSI) Debate in the Convention on Biological Diversity

Although there is recognition that DSI has advanced the conservation and sustainable use of biodiversity, there is still some debate on its use within the CBD. The Ad Hoc Technical Expert Group (AHTEG) on synthetic biology initially raised the issue of ABS in its 2015 meeting. At the 13th Conference of Parties (COP 13) to the CBD in 2016 in Cancun, Mexico, Namibia wanted a COP Decision recognising that DSI is subject to the third objective of the CBD. AHTEG work on DSI then proceeded from that COP.

CBD COP 14 in 2018 in Sharm El-Sheikh, Egypt compromised with Decision 14/20. Para. 12 specifically requests the open-ended working group established under decision 14/343 to consider the outcomes of the extended AHTEG and to make recommendations to the COP 15 on how to address DSI on genetic resources in the context of the Post-2020 Global Biodiversity Framework. The decision noted the divergence of views of Parties on this matter and decided to establish a science and policy-based process including convening the AHTEG.

In the CBD meetings in Geneva, Switzerland on 14–29 March 2022, a draft COP recommendation was developed that proposed a Global Multilateral Benefit-Sharing Mechanism. However, there is a need to update the Global Strategy for Plant Conservation (GSPC) on DSI as it appeared that at that time, the Subsidiary Body on Scientific, Technical and Technological Advice (SBSTTA) 24 and Subsidiary Body on Implementation (SBI) meetings still had not received any recommendation relevant to DSI that came from the groups that were advocating for an update to the GSPC.

Conclusion

Access and benefit-sharing is not such a problem that cannot be overcome as most questions and issues can be resolved by consulting the appropriate ABS regulation of the country where the research will be conducted. While some researchers and organisations may find ABS regulations difficult and cumbersome, these are meant to protect resource provider countries and communities, and should be complied with. Perhaps a global multilateral benefit-sharing mechanism could help deal with the challenges of ABS when a full DNA barcode library is established. It is also possible to help address the DSI issue when tied up with DNA barcoding in the updated GSPC.

Considerations in Permits to Collect Specimens

Sahlee B. Barrer

The collection of specimens and genetic resources is a very important issue that is regulated by both global environmental agreements and national legislation. These include the Convention on International Trade in Endangered Species in Wild Fauna and Flora (CITES), which provides for the protection and regulation of rare species, and thus has implications on the collection, export and import, and access to specific species.

Another relevant environmental agreement is the Nagoya Protocol on Access to Genetic Resources and the Fair and Equitable Sharing of Benefits Arising from their Utilization to the Convention on Biological Diversity. The protocol entered into force in 2014 and was ratified by 129 Parties to the CBD in February 2021. The collection of species and the analysis of their DNA sequences is an important part of the research process and collaboration between biodiversity-rich countries and those countries who seek access to such biodiversity. Each Party has thus taken appropriate legislative, administrative, or policy measures regarding the utilisation and protection of their own genetic resources.

It is therefore mandatory for organisations to determine the procedures and permits necessary to facilitate field work and specimen collection as required by national and local authorities. Each ASEAN Member State will have its own set of regulations regarding the collecting and handling of biological specimens. The permitting process and the organisations and agencies involved will depend on who has jurisdiction over the genetic resources. Location in a protected area, ASEAN Heritage Park, ancestral domain, or area with distinction as a World Heritage Site, Ramsar site, or Key Biodiversity Area, will provide additional layers of protection and other permitting requirements. These will have implications on the schedules for specimen collection and a project's timetable of activities.

Conceptual Framework and Workflow of DNA Barcoding

Ian Kendrick C. Fontanilla

DNA barcoding is a method that makes use of a short segment of the genome for rapid and accurate identification of an unknown individual to known species based on an available database (Hebert & Gregory 2005). In the past two decades, the use of molecular data for identification has gained momentum due to limitations posed by traditional taxonomy. These include the phenotypic plasticity and genetic variability exhibited by certain taxa, the presence of cryptic taxa that resemble each other superficially to be mistaken for the same taxon, the ambiguity of morphological keys when applied to specific life stages or sex of different taxa, and the high level of expertise required to identify taxa (Hebert et al. 2003a).

Standard regions to be used for barcoding purposes could vary across taxonomic groups. For animals, the standard marker is the cytochrome c oxidase subunit I (COI) gene (Hebert et al. 2003b), although other markers such as the small subunit ribosomal RNA (SSU rRNA) gene have also been utilised for other animals such as nematodes (Floyd et al. 2002). In fungi, the preferred marker is the internal transcribed spacer (ITS) region, while for bacteria they are the 16S rRNA and the chaperonin-60 (cpm60) genes (Tindall et al. 2010; Links et al. 2012). In plants, the preferred markers are the ribulose-1,5-bisphosphate carboxylase (*rbcL*) and maturase K (*matK*, Figure 1) genes as well as the *trnH-psbA* spacer and internal transcribed spacer II (ITS2) (Kress 2017).

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CAACCCCTTCGCTACTGGGTAAAAGATGCCTCTTCTTTACATTTATTACGGT
TCTTTCCTCCACGAGCATTTTAATTGGAATAGTCTTATTATTCCAAAGAAGCTC
CATTCCTTTTTTTTCAAAAAGGAATCCAAGATTATTATTGTTTCTATATAAT
TCTCATGTATATGAATATGAATCCATCTCTTTTTTCTCTGTAACCAATCTT
CTCATTACGATCAACATCCTCTGGAGTCCCTTGTTGAGCGAATATTTTTCTA
TAGAAAAGTCGAAACATCTTGTGCAAGTCTTTACTAATGATTTTCAGGACATC
TTATGGTTGTTCAAGGATCCTTTTATGCATTATGTTAGATATCAAGGAAAAAT
TCATTCTTGCCTTCAAAAAGATACGCCCTTCTGATGAATAAATGGAAATATTA
CCTTGTCAAATTTATGGCAATGGCATTTTCATGTGTGGTCTCAACCAGGAATG
TCTCATATAAATCACTTAGACAAGCACTCTATCAACTTTCGGGCTATCTTT
CTAGTGTGCGACTAAATCCTTTGGTGGTACGGAGTCAAATGCTAGAAAATTC
ATTTTTAATAGATAAATACTGTGAAGAAGGTTGATACAACCGTTCCAATTAGT
CATCTGATTTGGATCATTGACTAAGGCACGGTTTTGTAATCAATTAGGGCATC
CCATCAGTAAGCCGACCTGGGCTGATTCGCGGGATTCTCATATTATCGACCG
ATTTGCGCGT
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Figure 1. An actual *matK* DNA barcode of *Chisocheton pentandrus* (Blanco) Merr. subsp. *pentandrus* (BOLD Process ID VPSEA062-22)

Species concepts and DNA barcoding

DNA barcoding invokes two species concepts: phenetic and evolutionary or phylogenetic. The phenetic species concept follows the principle of numerical taxonomy where a species is viewed as individuals having shared similarities following established threshold values (Strickberger

2000). In DNA barcoding, this is measured by the overall similarity of the nucleotide sequences being compared. One application that can be used to search for the closest match for an unknown individual is the Basic Local Alignment Search Tool (BLAST) available in GenBank (Altschul et al. 1990).

The evolutionary or phylogenetic species concept defines a species as a distinct lineage that has been evolving separately from other lineages that allowed them to develop their own tendencies and characteristics (Strickberger 2000). In DNA barcoding, this can be represented by phylogenetic trees where placement of the unknown individuals relative to the other known species are determined.

The barcoding gap and the threshold values

When individuals of the same species are evaluated based on a specific DNA barcode marker, it is expected that differences in some nucleotide positions will be observed as a consequence of genetic variation. This intraspecific variation, however, is implied to be smaller when compared to individuals from different species. Consequently, if evaluating a group of individuals from different species from, say, a taxonomic genus or family, then the largest intraspecific variation is expected to be smaller than the smallest interspecific variation. This observation is used as a premise to identify the threshold value for determining if individuals belong to the same species or not. For instance, if two individuals have a genetic difference for a particular DNA barcode that is less than the threshold value, then they are assumed to be of the same species; otherwise, they belong to different species (Meyer & Paulay 2005). Different taxonomic groups may have different threshold values depending on the marker used. For the COI gene, for example, the threshold value for birds is 2.7 per cent (Hebert et al. 2004), for cowries it ranges from 1.99 to 2.85 per cent (Meyer & Paulay 2005), and for fishes it ranges from 3 to 3.5 per cent (Ward et al. 2009). In reality, however, overlaps between the intra- and interspecific variations may occur, which could lead to false positives where individuals from the same species with deep genetic divergences are mistakenly classified as two different species, or false negatives, where individuals from two different species but with genetic differences that are smaller than the threshold value are lumped together as one species (Meyer & Paulay 2005).

DNA barcoding workflow

For DNA barcoding to work, a comprehensive and readily accessible database should be available especially for the taxonomic group in question. For that to happen, there is an urgent need to first populate the database with barcode sequences and other available data from as many representatives of as many species as possible for the taxonomic group. It is therefore imperative that molecular biologists and taxonomists work hand in hand to generate such a database, where the taxonomists help identify the specimens to be barcoded and provide other taxonomic information, while the molecular biologists obtain the DNA barcode sequences.

The workflow for DNA barcoding (Figure 2) is as follows. First, the specimens must be identified correctly. Then, morphological and other information about the specimen must be gathered. These include the taxonomically important morphological characteristics that need to be identified or measured, the date and place where the specimen was collected, who collected the specimen and who identified it, and if applicable, where the specimen is deposited and its accession number. Photo documentation is required in cases where the specimen cannot be brought to a herbarium or museum for storage. Next, biological samples will be obtained and used as a source for DNA extraction, from which the DNA barcode sequence will be determined through polymerase chain reaction (PCR) amplification and sequencing. Finally, all these data must be deposited to an accessible database such as the Barcode of Life Data System (BOLD).

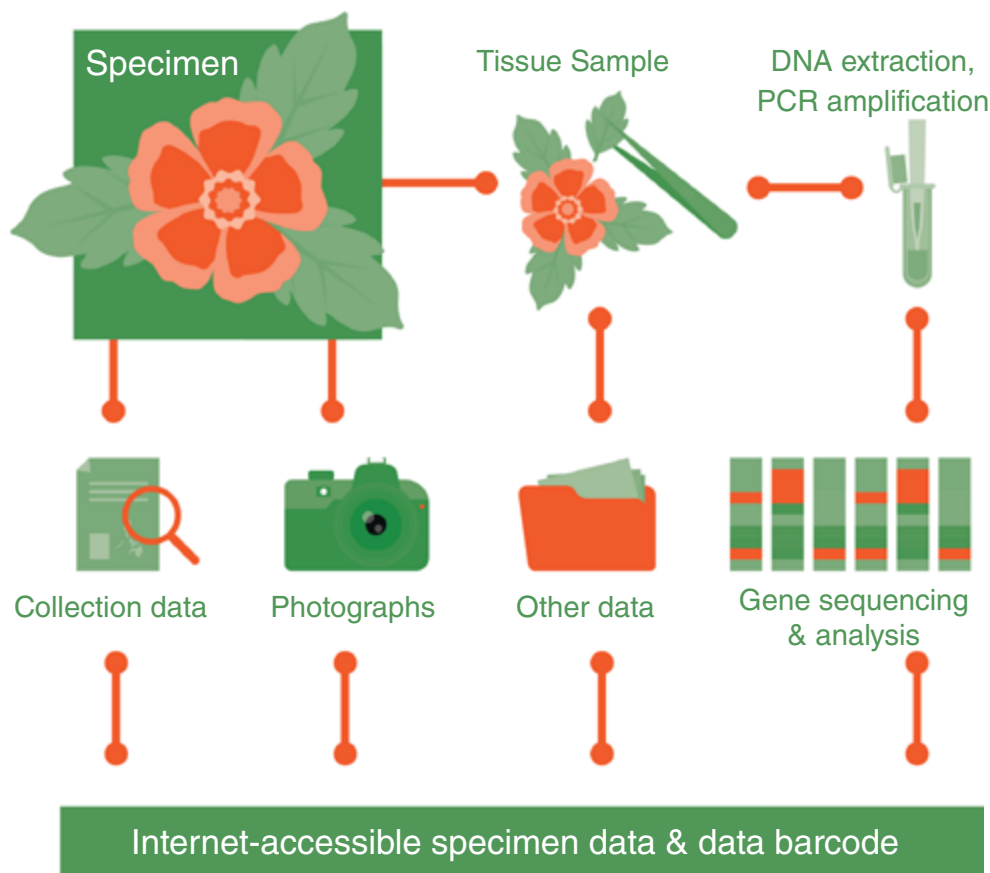


Figure 2. DNA Barcoding Workflow

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Collecting Plant Material for DNA Barcoding and Herbarium Voucher Preparation

David Justin R. Ples and Abigail L. Garrino

Plant collections are assortments of preserved specimens used for scientific research. They play an important role in botanical inventories, specimen identification, phylogeography, plant physiology, and novel plant studies. The process of plant collection is the first step in the preparation of herbarium voucher specimens. These voucher specimens provide insight into the composition of plants in their site locality, how they live, and interact with their natural environment. By collecting plant specimens, the preservation of floristic information is ensured and can be passed down through generations of researchers.

The genetic material used for DNA barcoding comes from plant material (typically young/fresh leaves) collected in the field, here referred to as ‘DNA vouchers.’ In addition to DNA vouchers that are directly involved in extraction and sequencing, separate ‘herbarium vouchers’ should also be collected as proof of the collected plant’s identity.

Materials for general specimen collection

- For spotting plant materials: binoculars
- For taking samples: pruning shears or pole cutter, latex gloves
- For tagging collected material: parchment paper, crochet threads, hole puncher
- For taking field notes: field notebook, pencil, eraser, sharpener, GPS
- For photographing plants: camera, any plain flat object to use as a background (e.g. illustration board, folder)

Materials specifically for DNA voucher collection

- For storage: teabags or small paper envelopes or ziplock bags, silica gel, a container such as a jar or a box that can fit several tea bags or envelopes

Materials specifically for herbarium voucher collection and temporary storage

- For field collection and processing: industrial plastic bags, old newspapers, 70% ethyl or denatured alcohol, straw ball or rope

Pre-collection preparations

1. Creating collection tags: Cut the parchment paper into rectangles (recommended size: 5 cm x 2.5 cm). Punch a hole on one end and tie a short length of crochet thread through it to loop the tag around a branch or twig.
2. Field notes and datasheets: Take note of the collector names or initials and numbers, tentative plant identifications, and the location of the collection.

Collecting DNA vouchers

1. Select 3–5 juvenile leaves from the tip of a branch. Make sure that the leaves collected are clear of any dirt, debris or other material.
2. Write out a collection tag, indicating the collector's name and plant collection number. Initial identifications and other noteworthy details (colours, odours, habitat description, and others) should be written in the field notebook.
3. Place the leaves together with the collection tag in the paper envelope or small ziplock bag. Add enough silica gel (about 1/2 of the envelope or bag) to keep moisture from building up until the specimen can be processed for DNA extraction.



Preparing herbarium field collection number.

Photo by Pamela Q. Reblora

Collecting herbarium vouchers

1. Before collecting an herbarium voucher from the same plant:
 - a. Write out a collection tag indicating the collector's name and plant collection number. Make sure this matches the tag used for the DNA voucher.
 - b. Photograph the whole plant, if possible, to capture its habit, branch architecture, and other characteristics. Then take close-up photos for details of the leaves, stems, flowers, and fruits. Adjust camera settings to darken the background of these images, or else photograph the plant parts against a dark background (e.g. the dark side of an illustration board, a black cloth, and others). Placing a ruler or an object with known measurement (e.g. a coin) next to plant parts allows them to be accurately measured later on.
 - c. Record initial identifications and other important information in the field notebook. Engage your senses and write down valuable characters which may not be captured on camera, such as leaf texture, flower scent, and others.



Preparing herbarium specimen.

Photo by Martin A. Palis

2. Collect a twig containing both vegetative (stems and leaves) and reproductive (flower, fruit) parts. Flowers and fruits should be collected when available as their characters are considered more stable and often necessary to identify the plant down to species level.
 - a. Cut below or after the point of attachment to the main stem, as certain characters necessary for identification such as stipules or swollen nodes may be missed by cutting higher up.
 - b. The specimen should be small enough to fit within the boundaries of a standardized herbarium sheet. For some groups such as palms, a large compound leaf may have to be cut into pieces, with each separately tagged, mounted, and labeled on multiple herbarium sheets.
 - c. For other groups with bulky fruits, cross-sections may have to be made so that the specimen can be stored flat with others.
3. Press collected specimens.
 - a. Decide whether to press individual specimens in the field or process them in bulk back at camp. The former can be done when sampling only a few targeted or opportunistic specimens, while the latter should be done when systematically sampling a larger area, to save time and move efficiently. When pressing in the field, collections should be carried in a mobile or field press; if waiting to press at camp, collections can be temporarily carried in a large industrial plastic bag.

- b. When pressing can begin, place each collected specimen inside an individual fold of an old newspaper.
 - c. The vegetative portions should be arranged during pressing to properly display leaf arrangement, as well as prominent or diagnostic characters such as stipules, lenticels, thorns, or tendrils. Some leaves should show the adaxial (upper) surface, while others can be turned or twisted to make the abaxial (lower) surface visible.
 - d. Use a permanent marker to write details (e.g. collector's name, collection number, initial ID) on the newspaper to help find particular collections faster.
 - e. Stack the newspapers containing the specimens and tie them into a bundle with straw or rope. The knot should neither be too loose (to avoid collections from falling out) or too tight (to avoid crushing or deforming the contents).
4. Place the bundle of pressed collections inside a thick industrial plastic bag and douse it with an ample amount of 70% ethyl or denatured alcohol. Carefully turn the pile to make sure that all sides are evenly soaked. Knot the plastic tightly and check for any leaks or spills.



Pressing herbarium specimens in the field.
Photo by Pamela Q. Reblora

Drying, Mounting, and Filing of Herbarium Vouchers

David Justin R. Ples and Abigail L. Garrino

Herbarium vouchers are common and essential tools for botanists. They are used for the identification, classification, and preservation of many different plant species. Without proper herbarium vouchers, it would be extremely difficult, if not impossible, to correctly identify a plant's genus and specific epithet. Therefore, the proper handling of each specimen should be practiced from drying, mounting, and filing inside the herbarium storage cabinets.

Specimens doused in alcohol can be kept in storage for several months, crucial to long-term fieldwork. Once transported back to the herbarium, pressed specimens can then be dried, mounted, and accessioned.

Preparing specimens from the field

Supplies and equipment

- Old newspaper
- Wooden herbarium press
- Cardboard
- Corrugated iron sheets
- Rope or binding straps
- Specimen dryer
- Herbarium mounting sheets
- Wood glue or gum tape
- Brush and forceps
- Herbarium label
- Bristol folders

1. Incoming specimens may go through various treatments before being integrated into an herbarium collection. At a minimum, specimens drenched in alcohol must be dried before mounting.
2. Where facilities are available, incoming specimens may also go through deep freezing (at -18 to -25°C , for at least 48 hours) or microwaving (for 75–120 seconds). The latter is only for dry specimens and for plants without known volatile oils, as this process may affect the chemistry, pollen, and DNA.
3. Poisoning specimens using toxic chemicals such as mercury, although once a popular practice, is no longer regarded as a safe method and is not recommended.

Drying specimens

1. Carefully remove the collected specimens from the industrial plastic bag, letting excess alcohol drip off. Replace all newspaper folds with a drier stack of collections.
2. Begin placing collections into an herbarium press.
 - a. The first of the two wooden frames should act as the base of the press.
 - b. Place down a sheet of cardboard, followed by a specimen in a newspaper fold. Press this down with a second cardboard sheet. Continue to add stacks this way,

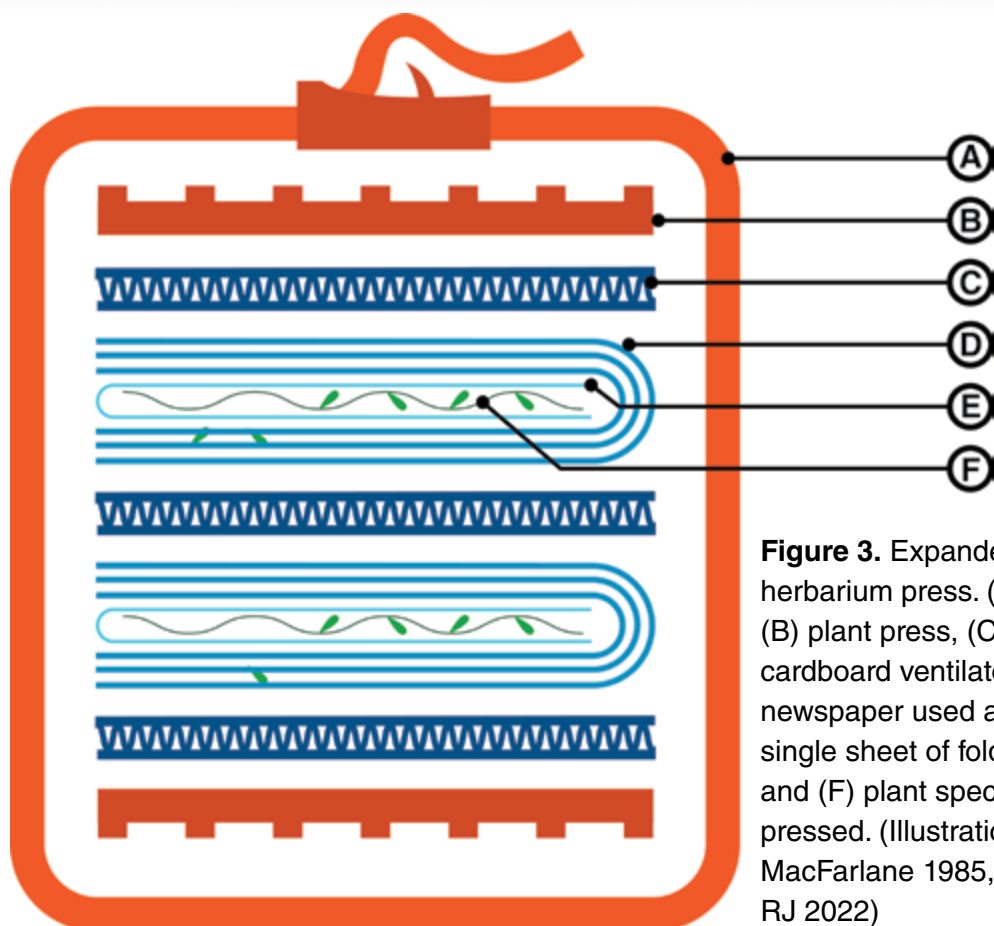


Figure 3. Expanded view of an herbarium press. (A) binding strap, (B) plant press, (C) corrugated cardboard ventilator, (D) thick newspaper used as a blotter, (E) single sheet of folded newspaper, and (F) plant specimen being pressed. (Illustration after MacFarlane 1985, redrawn by Rey, RJ 2022)

with each group of cardboard–specimen–cardboard separated by a corrugated iron sheet (Figure 3), which will allow hot air from the dryer to pass through the press evenly.

- c. Once all specimens have been placed or the press is about a foot high, use the second wooden frame to close the bundle, and secure it using the binding straps or *abaca* (hemp) rope. Additional specimens after the first foot should be placed in a separate press, since a thick press makes the drying process less effective and the bundle more difficult to manage.
3. Place the presses inside the herbarium dryer for heating.
 - a. The herbarium dryer temperature should be about 55–60°C.
 - b. The process may take from a couple of days to about a week, depending on the thickness of the press and how many hours a day the dryer can be left on.
 - c. Unplug the dryer after office hours to prevent untoward incidents and loss of collected material.

Mounting specimens

1. Once specimens have been thoroughly dried, they should be mounted onto herbarium sheets, standardly 29 cm x 43 cm.
 - a. Before mounting the specimen, place the specimen at the center of the herbarium sheet and find the best orientation for it. Ensure that the collection tag is still tied to the specimen; there are no parts hanging off the edges; and there is ample space around the corners for labels, accession stamps, and packets (Figure 4).

- b. Mounting involves applying a conservative amount of wood glue or gum glue to the parts of the dried specimen that would be touching the herbarium sheet when it is laid flat. The specimen should be firmly attached to the sheet while also being accessible for researchers who may need to inspect and manipulate certain characters manually for research. Use small sandbags or any heavy object to apply weight to the specimen while the glue is drying.
 - c. Specimens may also be strapped down with linen tape or sewn onto the herbarium sheet.
 - d. As mentioned in the section on Collecting Herbarium Vouchers (page 30), a series of several mounting sheets may be necessary for a single large specimen (for some groups such as palms). Some specimens (e.g., *Arecaceae*, *Zingiberaceae*) may require special handling.
2. Store small loose parts in paper envelopes or packets, also attached to the sheet with glue.
 3. Once the specimen and packet have been firmly attached, place the herbarium label in one corner (usually the lower right). The label should include all pertinent details such as the herbarium name, collector name, collection number, and date, locality, elevation, common or vernacular names, any recorded uses or cultural value, and others.

Accession of mounted specimens

1. After labels have been affixed to the mounted voucher, give it an accession number before adding the specimen to an herbarium's collection. This should be recorded by the herbarium in a logbook or digital spreadsheet, with the number stamped onto the herbarium sheet (usually on the upper right).
2. Mounted and accessioned specimens are placed in heavy bristol folders called 'genus covers' (standardly 61 cm x 42 cm) and placed together with other specimens of the

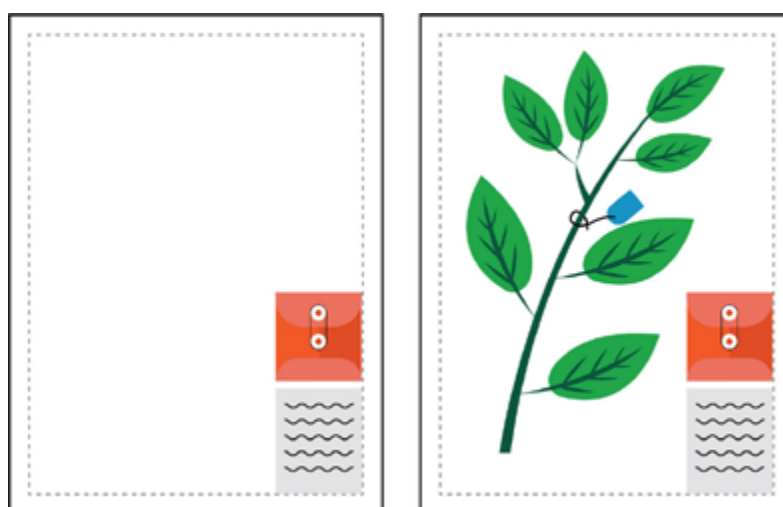


Figure 4. Herbarium mounting sheets with labels, paper packets, and specimen positioned free from the margin (Illustration after Bridson & Forman 1989).

same taxonomic level in herbarium cabinets or compactors. Herbarium collections are sorted by family, genus, and species, typically following the most recent phylogenetic system recognised by the botanical community (e.g. APG 2016). This allows phylogenetically related families and genera to be placed close to one another, facilitating identification by matching morphological characters.

3. Additional sorting may be necessary if geographic regions are recognised in the collection. Type specimens (the first collections of a species, upon which the formal description is based) are usually placed in separate herbarium cabinets.
4. The herbarium where a collection is first deposited should facilitate the distribution of replicates to other herbaria, ensuring that material is preserved in case of untoward incidents such as fires or insect outbreaks.

References

- APG (Angiosperm Phylogeny Group) (2016). An update of the Angiosperm Phylogeny Group classification for the orders and families of flowering plants: APG IV. *Botanical Journal of the Linnean Society* 181(1): 1–20.
- Blanco, M.A., Whitten, W.M., Penneys, D.S., Williams, N.H., Neubig, K.M., & Endara, L. (2006). A simple and safe method for rapid drying of plant specimens using forced-air space heaters. *Selbyana* 27(1): 83–87.
- Bridson, D. & Forman, L. (eds.) (1989). *The Herbarium Handbook*. Royal Botanic Gardens, Kew, United Kingdom. 334p.
- MacFarlane, R.B.A. (1985). *Collecting and Preserving Plants for Science and Pleasure*. Arco Pub., New York. 184p.

Imaging and Photography

David Justin R. Ples and Abigail L. Garrino

Digitisation allows researchers and other interested parties to access herbarium specimens remotely, which is important when resources (e.g., funds for travel, ample time) are unavailable or circumstances prevent viewing (e.g., during a global pandemic).

Considerations for specimen photography include:

- Capture devices (e.g., digital cameras, flatbed scanners)
- Lighting and reduction or elimination of shadows
- Visibility of character traits (e.g., presence of trichomes, texture of bark or leaves)
- Readability of text (e.g., on herbarium labels, collection tags)
- Inclusion of packeted material
- Inclusion of a colour reference chart

Once vouchers are photographed, all written information must be keyed into digital spreadsheets to be uploaded together with the images on the herbarium's website or other online databases. Examples of articles that describe options for equipment, camera settings, lighting set-up, and efficient workflows in detail include:

- Harris, K.M., & Marsico, T.D. (2017). Digitizing specimens in a small herbarium: A viable workflow for collections working with limited resources. *Applications in Plant Sciences* 5(4): 1600125.
- Takano, A., Horiuchi, Y., Fujimoto, Y., Aoki, K., Mitsuhashi, H., & Takahashi, A. (2019). Simple but long-lasting: A specimen imaging method applicable for small-and medium-sized herbaria. *PhytoKeys* 118: 1–14.
- Tulig, M., Tarnowsky, N., Bevans, M., Kirchgessner, & A., Thiers, B.M. (2012). Increasing the efficiency of digitization workflows for herbarium specimens. *ZooKeys* (209):103–13.



Taking photos of specimens in the field.

Photo by Pamela Q. Reblora

Pollia thyrsoiflora (Blume) Steud.
Photo by Edwino S. Fernando





PART 2

Laboratory
Processes and
Activities

Working in a molecular laboratory involves following various protocols for the safety of personnel and for preventing contamination of samples being analysed. For DNA barcoding practices, it is vital that working reagents and samples are free of contamination to save time and resources. Personnel should follow all safety precautions for protection from biological, physical, and chemical hazards that may be present in the laboratory.

Consider the following precautions while working in a molecular laboratory:

1. Maintain laboratory cleanliness and orderliness. A clean laboratory lessens health hazards for personnel working in the laboratory.
2. Always disinfect common surfaces such as laboratory benches, refrigerator handles, micropipettes, office tables within the laboratory, doorknobs and others using appropriate cleaning solutions, such as 70% alcohol or any disinfectant, before and after use.
3. Immediately wash and clean glassware after use.
4. Legibly label all reagent bottles with complete name and date of preparation.
5. Clearly label all samples and stock them according to assigned refrigerators. Always ask the laboratory manager for information regarding sample storage to avoid confusion with other personnel working with different samples.
6. Prevent contamination in DNA extraction particularly when dealing with two or more putative plant species for one time session of extraction.
7. In handling nucleic acid stain, carefully get and dispense using appropriate micropipettes and dispose of tips in a separate labeled container properly labeled 'nucleic acid stain.'
8. Always ask the laboratory head or principal investigator for inquiries and report any accidents immediately for proper action.
9. Dispose all solid, liquid, and other types of wastes according to the guidelines set by your environmental agencies.
10. Do not eat, drink, or bring any food items inside the laboratory.
11. Post hotlines in laboratory bulletin boards such as the local fire department, nearest hospitals, school administration offices and other relevant contact numbers in case of emergencies.

Personal Protective Equipment (PPE)

Clean laboratory gowns and gloves are compulsory in every molecular laboratory. Wear safety glasses and masks if demanded by specific laboratory protocols. Laboratory personnel should have their own personal laboratory gown. Laboratory gowns are for exclusive use only within laboratories and should be taken off when exiting. Contaminated gloves should be disposed of and replaced to prevent cross-contamination of common surfaces.

Potential sources of hazards

Physical

Physical hazards include ergonomic hazards, ionising and non-ionising radiation, and noise. Ergonomic hazards involve repetitive routine physical activities that may cause physical injuries such as musculoskeletal problems. Improper posture may also cause back pains and discomfort.

Exposure to radioactive sources can pose health risks to workers. Exposure to non-ionising radiation such as heat lamps, infrared lasers, ultraviolet (UV) transilluminators, and black lights, may cause eye and skin irritation.

Continuous, intermittent, and impulsive sound levels from 80–130 decibels A (dBA) can cause irritability, hearing loss, and tinnitus (i.e., ringing in the ears); reduce concentration and productivity; and increase errors in the laboratory, among others.

Instrument Hazards

Instrument hazards include potential injuries that can be acquired from sharp instruments and potential thermal burns.

1. Autoclaves and sterilisers - operated at very high temperatures. Personnel should be properly trained to handle and sort hot sterilised items and sharp materials from the autoclave or steriliser.
2. Centrifuges - operated at very high speeds. Can cause serious injury or even death to personnel if not operated properly. Personnel should be trained to follow good work practices in any molecular work utilising centrifuges.
3. Compressed gases - a gas or a mixture of gases with various components having an absolute pressure exceeding 40 pounds per square inch. Cylinders with compressed gases may be toxic, flammable, oxidising, corrosive, inert or explosive.
4. Cryogenics and dry ice - are substances used to produce very low temperatures. Exposure to these agents may cause cold contact burns, asphyxiation, explosion due to abrupt change in pressure, and explosion due to chemical reactions.

Chemical Hazards

Use the Material Safety Data Sheet (MSDS) to determine if toxic and hazardous components are present in some reagents. Wear gas masks or work in a fumehood with complete PPE when handling highly volatile reagents or if chemical reactions will generate toxic by-products.

Fire Hazards

Laboratories with solvents in any quantity have the potential for flash fires, explosions, quick spread of fire, and burns. Presence of fuel load and other hazards increases the severity of injury.

Common Equipment in a Plant DNA Barcoding Lab

Charles Anthon E. Cadorna

A plant DNA barcoding laboratory must be in a closed physical space and separated from offices and common areas. Several workspaces must be dedicated to a specific processing step to prevent cross-contamination. If working in a single room, ensure physical and spatial separation of equipment and consumables and unidirectional workflow to prevent contamination.

The photos below show the basic equipment needed to conduct genomic DNA extraction, assessment of DNA quality, polymerase chain reaction (PCR) amplification, and verification of amplification success by agarose gel electrophoresis.

Common equipment and their uses in a plant DNA barcoding laboratory



Thermal cycler

Also known as thermocycler or PCR machine; a common molecular biology laboratory equipment used to amplify segments of DNA via the PCR.



Centrifuge

An equipment that uses centrifugal force to facilitate separation during the different phases of extraction.



Homogenizer

Equipment used for mechanically breaking down plant tissue into smaller pieces. In this particular model, the samples are placed in a microcentrifuge tube along with metal beads. Mechanical breakdown is achieved by vibrating the tube at high frequencies.



Micropipettes

Used to transfer precise, very small volumes of liquid.



Spectrophotometer or Fluorometer

Used to assess the quantity and quality of isolated DNA.



Gel electrophoresis set-up

A set of equipment used to separate DNA fragments according to their size.



Gel imaging and documentation system

This equipment has an ultraviolet (UV) transilluminator and a camera to visualize and image gels after electrophoresis.



Vortex mixer

Used to mix small amounts of liquid rapidly.



Heat block or Digital Dry Bath

Used for incubation during DNA extraction.



Autoclave

Used to sterilise tubes and pipette tips.



Microwave

Used to melt agarose for gel electrophoresis.

Total Genomic DNA Extraction from Plant Samples

Jessica D. Rey and Charles Anthon E. Cadorna



Extracting DNA from plant samples.



Photos by Patricia S. Leuterio

DNA extraction is a basic technique in molecular analysis. Plant DNA can be sourced from different plant parts (e.g. leaves, flowers, stems and roots). The DNA extraction methods involve three main steps: (1) cell lysis, (2) removal of non-DNA material (e.g. lipids, RNAs, and proteins), and (3) precipitation or separation of the DNA. Extracted DNA is then used as a template for DNA amplification. The DNA extraction from plant samples used in this manual follows the protocol of the DNeasy Plant Mini Kit by Qiagen, Inc.



DNeasy Plant Mini Kit

Materials

- Analytical balance
- Microcentrifuge tubes (2.0 ml and 1.5 ml)
- Tube racks
- Sterile tips
- Pipettors
- Dry bath
- Microcentrifuge
- Homogenizer
- Steel homogenizer beads
- Plant tissue (e.g., leaves)
- Qiagen® DNeasy Plant DNA Mini Kit
- Crushed ice

Procedure

1. Place the sample material (≤ 20 mg silica-gel dried leaves) into a 2 mL safe-lock microcentrifuge tube together with 4 pieces of stainless steel homogenizer beads. Homogenize the sample for approximately 5 minutes at 30 Hz.
2. Add 400 μL Buffer AP1 and 4 μL RNase A stock solution (100 mg/mL) and vortex vigorously.
3. Incubate the mixture for 10 minutes at 65°C. Mix 2–3 times during incubation by inverting the tube.
4. Add 130 μL Buffer P3 to the lysate. Mix and incubate for 5 minutes on ice.
5. Centrifuge the lysate for 5 minutes at 20,000x g (14,000 rpm).
6. Pipet the lysate into the QIAshredder Mini spin column (lilac) placed in a 2 mL collection tube, and centrifuge for 2 minutes at 20,000x g (14,000 rpm).
7. Transfer the flow-through fraction from Step 6 into a new tube without disturbing the cell-debris pellet.
8. Add 1.5 volumes of Buffer AW1 to the cleared lysate and mix by pipetting. For example, to 450 μL lysate, add 675 μL Buffer AW1.
9. Pipet 650 μL of the mixture from Step 8, including any precipitate that may have formed, into the DNeasy Mini spin column placed in a 2 mL collection tube.
10. Centrifuge for 1 minute at $\geq 6000x$ g (8000 rpm) and discard the flow-through. Repeat this step with the remaining sample.
11. Place the DNeasy Mini spin column into a new 2 mL collection tube, add 500 μL Buffer AW2 and centrifuge for 1 minute at $\geq 6000x$ g (8000 rpm). Discard the flow-through and reuse the collection tube in Step 12.
12. Add another 500 μL Buffer AW2 to the DNeasy Mini spin column, and centrifuge for 2 minutes at 20,000x g (14,000 rpm) to dry the membrane.
13. Transfer the DNeasy Mini spin column to a 1.5 mL microcentrifuge tube. Transfer the spin column from the collection tube carefully, so the column does not come into contact with the flow-through.
14. Add 100 μL of Buffer AE to the center of the DNeasy Mini spin column membrane for elution. Incubate for 5 minutes at room temperature, and then centrifuge for 1 minute at $\geq 6000x$ g (≥ 8000 rpm). Repeat this step.
15. Discard the DNeasy Mini spin column and keep the microcentrifuge tube with the eluted DNA.
16. Use the purified DNA immediately in downstream applications or store at -20°C.

DNA quality and quantity check

To check the purity of nucleic acids, DNA or RNA is usually estimated based on the absorbance maxima, which is a ratio between absorbance at 260 and 280nm. At this range, the highest purity level of DNA is ~ 1.8 , while the RNA is ~ 2.0 .

Another way to check for the quality of DNA is by gel electrophoresis, where the DNA sample is loaded on a gel and run side by side with a known DNA marker such as lambda DNA with known size and purity.

Polymerase Chain Reaction Amplification

Rogel Victor D. Mendoza and Charles Anthon E. Cadorna



Preparing DNA for PCR.



Photos by Patricia S. Leuterio

Amplification of DNA involves the use of polymerase chain reaction (PCR), where the enzyme DNA polymerase is utilised to replicate a region of interest in the genome of an organism. In this procedure, the DNA region of interest (e.g. for plants, a particular gene like *matK* and *rbcL*) is amplified using specific primers. Below are some of the DNA barcode primers for plants from Fazekas et al. 2012:

rbcL

rbcLa-F - ATGTCACCACAAACAGAGACTAAAGC

rbcLajf634R - GAAACGGTCTCTCCAACGCAT

matK

matK3F_KIM - CGTACAGTACTTTTGTGTTTACGA

matK1R_KIM - ACCCAGTCCATCTGGAAATCTTGGTTC

Other components in PCR reaction are: (1) deoxynucleoside triphosphates (dNTPs), which will be the source of nucleotides for DNA synthesis; (2) PCR buffer, which provides suitable condition for the activity of DNA polymerase enzyme; and (3) divalent cation such as Mg²⁺ which are sometimes included in the PCR buffer. These components are mixed in a reaction tube and comprise the PCR mix. The concentration of each component is optimised to yield good results (excess or insufficient components might hinder amplification).

Materials

- Sterile 0.2 ml PCR tubes
- Tube racks
- Sterile tips
- Pipettors
- Centrifuge
- PCR machine
- DNA sample
- Ultra-pure water
- PCR buffer
- Primers
- Taq polymerase

DNA amplification is carried out in a PCR thermocycler. Temperature in the thermocycler changes in a defined manner to achieve the following: (1) *denaturation stage*, the temperature where DNA strands are denatured so that the DNA will be open for binding of primer; (2) *annealing stage*, the temperature optimised for the primer to bind in the specific target region; and (3) *extension stage*, temperature optimised for the addition of nucleotide to the primer by the DNA polymerase.

Procedure

1. Prepare a PCR master mix containing the following:

Component	25.0 μ l reaction	x reactions
Ultra-pure water	17.375 μ l	
PCR buffer	2.500 μ l	
10 μ M forward primer	1.250 μ l	
10 μ M reverse primer	1.250 μ l	
dNTPs	0.500 μ l	
Taq	0.125 μ l	
DNA template	2.000 μ l	

Note: Always include a negative control PCR reaction and an extra reaction to account for pipetting errors.

Program the thermal cycler using the following parameters:

Steps:

95°C, 2 mins

94°C, 30 s

54°C, 30 s

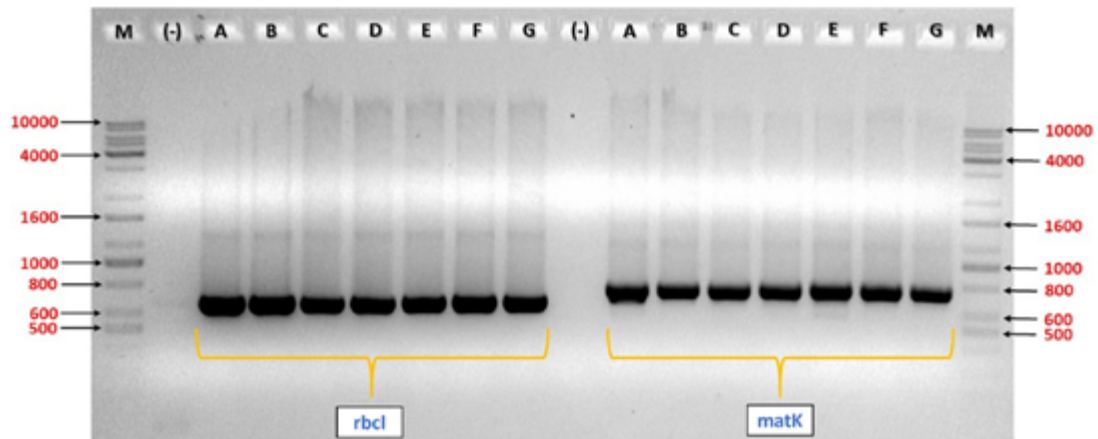
72°C, 1 min

35 cycles

72°C, 10 min

4°C, ∞

2. Assess the quality of PCR amplification by running 3 μ l of the reaction on a 1% agarose gel. The expected size of *rbcL* and *matK* PCR products are ~600 bp and ~800 bp, respectively. No bands should be present in the negative control reaction. An example of a successful amplification is shown below.



Sample PCR result for *rbcL* and *matK*

Lane 1: DNA ladder	Lane 7: Sample E	Lane 13: Sample C
Lane 2: negative control	Lane 8: Sample F	Lane 14: Sample D
Lane 3: Sample A	Lane 9: Sample G	Lane 15: Sample E
Lane 4: Sample B	Lane 10: negative control	Lane 16: Sample F
Lane 5: Sample C	Lane 11: Sample A	Lane 17: Sample G
Lane 6: Sample D	Lane 12: Sample B	Lane 18: DNA ladder

Labels for each of the columns in the sample PCR result for *rbcL* and *matK*

Reference

Fazekas, A.J., Kuzmina, M.L., Newmaster, S.G., & Hollingsworth, P.M. (2012). DNA barcoding methods for land plants. *Methods in Molecular Biology* 858: 223–252

Agarose Gel Electrophoresis

Jessica D. Rey, Charles Anthon E. Cadorna, and Rogel Victor D. Mendoza



Loading DNA for AGE.



Photos by Patricia S. Leuterio

Agarose Gel Electrophoresis (AGE) is a method used in experiments involving the separation of biomolecules (e.g. proteins, DNA, RNA) by their charge and size or length. This separation is achieved by running a current through the agarose matrix, with smaller DNA fragments moving faster than the larger DNA fragments towards the positively charged direction of the electric field. The separated DNA fragments are then stained using nucleic acid stain and visualised using UV light (Reece 2004). In this module, AGE is used to visualise the polymerase chain reaction (PCR) products and estimate their fragment length. Below is the protocol for preparation of a 1% agarose gel.

Materials and Equipment

- Agarose (molecular biology grade)
- 0.5x/1.0x Tris-Borate-EDTA (TBE) Buffer / 1.0x Tris-Acetate-EDTA (TAE) Buffer
- Nucleic acid stain such as ethidium bromide (EtBr)
- Gel loading dye
- 100 bp or 1 kb molecular ladder
- Electrophoresis set-up
- Gel casting materials
- Pipettors

Procedure

1. For 1% agarose gel, weigh 1.0 g of agarose and dissolve it in 100 ml of 0.5x or 1.0x TBE buffer. Alternatively, 1.0x TAE buffer may also be used. Heat the agarose solution in a microwave oven for 3 minutes. Allow the gel to cool for about 10 minutes. While cooling, prepare the casting tray and comb that will be used for molding the gel.
2. Upon cooling, add 1.0 μ l of EtBr and gently swirl until evenly mixed. Pour the melted agarose on the casting tray and place the combs. Make sure that there are no air bubbles in the gel. Allow the gel to set for about 10–20 minutes, indicated by the gel turning from transparent to translucent.

3. Remove the combs carefully once the gel has set. Submerge the gel in the electrophoresis tank containing 0.5x/1.0x TBE Buffer. Make sure that there are no air bubbles in the wells.
4. Prepare the PCR products for loading by adding 1.0 µl of gel loading dye, mix by pipetting. The gel loading dye increases the density of the PCR product, allowing it to sink to the well when loaded.
5. Load 2.0 µl of the 100bp or 1kb molecular ladder in the first well (*you may choose where to load the molecular ladder*). In the succeeding wells, load PCR products by pipetting them into the well. Make sure that no samples spill from each well.
6. Set the power supply voltage to 85–100 V. Allow the set-up to run for 30 minutes. After this, remove the gel from the tank and proceed to visualisation under the UV transilluminator.

Gel Extraction

Rogel Victor D. Mendoza and Charles Anthon E. Cadorna

The first purpose of performing Agarose Gel Electrophoresis (AGE) is to visualise the polymerase chain reaction (PCR) product. The second purpose is to separate the desired PCR product if multiple bands appear on the gel. Each band represents a PCR product of a particular size. In this case, the desired product is selected and excised from the gel, followed by an extraction procedure. This ensures that the PCR product that will be sequenced contains only the amplified fragments of the desired marker and all of them are of the same fragment size. DNA recovery is then checked through AGE. The gel extraction in this manual follows the protocol of the commercial gel extraction kit.

Materials and Equipment

- Gel solubilisation buffer
- Wash buffer (with absolute ethanol)
- Elution buffer
- Spin columns
- Collection tubes
- 2.0 ml tubes
- 1.5 ml tubes
- Microcentrifuge
- Pipettors
- Analytical balance
- Dry bath

Procedure

1. Excise the desired band from the agarose gel and place it inside a pre-weighed 2.0 ml tube. Weigh the excised gel using an analytical balance. Add a 1:1 wt/v ratio mg gel to µl of gel solubilisation buffer. Heat at 50–60°C for 10 minutes in a dry bath or until gel solids are no longer seen in the solution.
2. Transfer the dissolved sample into a spin column (maximum of 800 µl). Set and spin the microcentrifuge to its maximum speed for 1 minute. Discard the contents of the collection tube. Reuse the collection tube. (*If the dissolved sample is more than 800*

µl, repeat this procedure until all of the sample has been used up, utilising the same spin column and collection tube).

3. Add 100 µl gel solubilisation buffer into the spin-column. Spin at maximum rotor speed for 1 minute. Discard the contents of the collection tube. Reuse the collection tube.
4. Add 700 µl of wash buffer. Incubate at room temperature for 5 minutes. This ensures the removal of excess salts that may affect sequencing results. Spin at maximum rotor speed for 1 minute. Discard the contents of the collection tube. Reuse the collection tube.
5. Spin the empty spin-column at maximum rotor speed for 1 minute. This step ensures the removal of excess wash buffer and ethanol. Discard the contents of the collection tube. Discard the collection tube.
6. Transfer the spin-column into a fresh 1.5 ml tube. Apply 50 µl of elution buffer directly into the spin-column membrane. Incubate at room temperature for 5 minutes. Spin at maximum rotor speed for 1 minute. Remove spin-column and discard. Store the purified DNA sample in -85°C until used for sequencing.
7. To check if the desired fragment was successfully purified and recovered, run 1 µl of the sample in 1% AGE using the same protocol as the AGE experiment. The presence of a clean band with the same desired fragment length (estimate concentration by comparing with molecular ladder) indicates a successful gel extraction step.

DNA Sequencing

John Gregor A. Roño

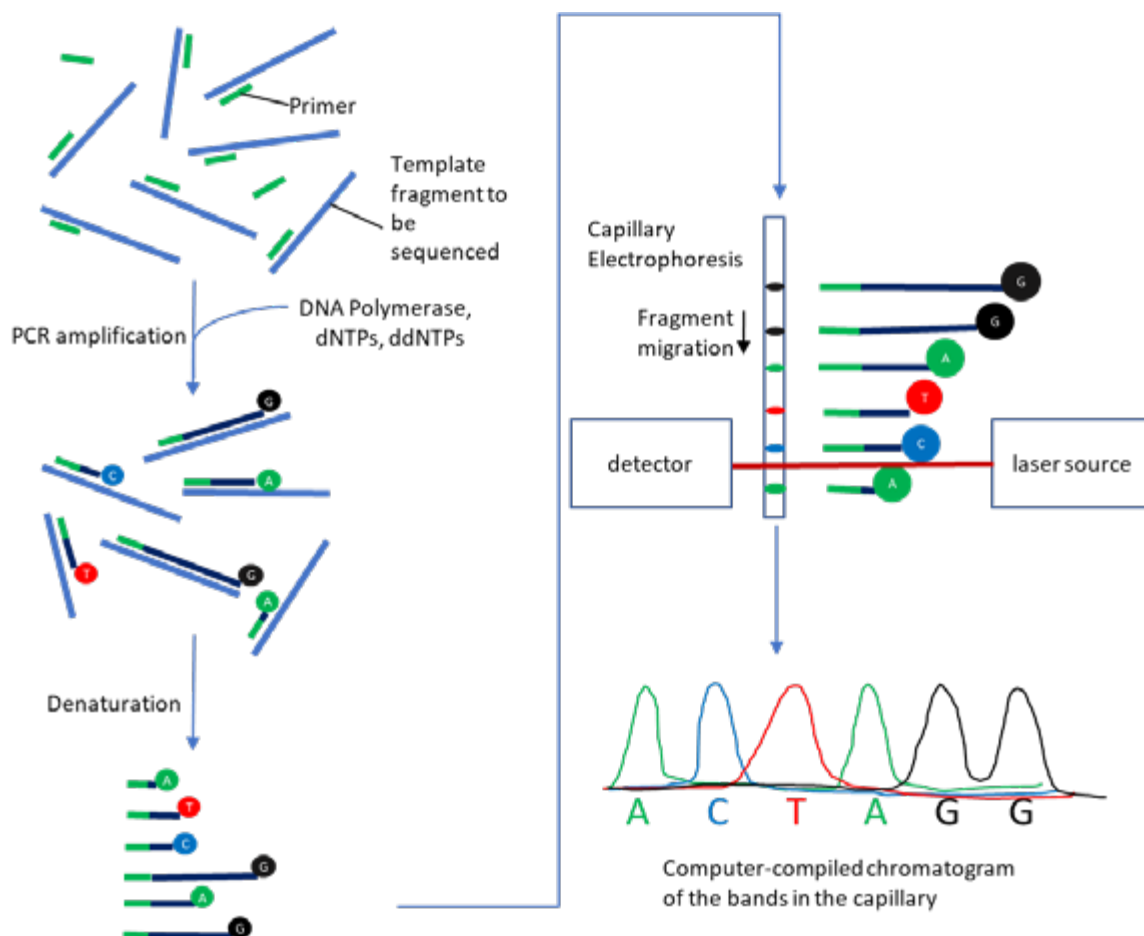
DNA sequencing employs the Sanger sequencing method or chain-termination method, which was developed by Frederick Sanger and colleagues in 1977 (Sanger et al. 1977). Traditionally, the method used a very large slab of agarose gel to perform the sequencing through Agarose Gel Electrophoresis (AGE). Modern developments automated this process and reduced the scale of the electrophoresis into a capillary tube (Karger & Guttman 2009), hence the term capillary sequencing that is also commonly used. This manual covers the general processes and concepts involved in Sanger sequencing. For DNA barcoding, it is more practical to send samples to a sequencing facility especially when handling a large number of samples.

A major step done before the actual Sanger sequencing is called cycle sequencing. This method amplifies the DNA template in a manner similar to a standard PCR but with two core modifications. First, only one primer is utilised in the reaction, making the amplification proceed with linear increase instead of being exponential. Second, fluorescence-labeled dideoxynucleotide triphosphate (ddNTPs) is added along with the regular deoxynucleoside triphosphate (dNTP) in the mixture. The nucleotide chain synthesis proceeds in a similar way in a standard polymerase chain reaction (PCR) amplification. When a ddNTP is added to the growing nucleotide chain instead of a dNTP, it prevents the addition of a succeeding nucleotide. This terminates the synthesis of the nucleotide chain, hence the term 'chain-termination method.' This process is random at different nucleotide sites. At the end of the amplification process, the sample solution will now contain a mixture of fragments with differing lengths

containing a labeled ddNTP at their 3' end. A clean-up step is then performed to remove the excess primers and nucleotides that may interfere in the succeeding Sanger sequencing.

The sample is then loaded into an automated Sanger sequencer. Following the principle of the AGE, the mixture of fragments in the sample is run through a gel and are separated according to fragment length inside a long, thin capillary tube. Recall that a certain fragment length is associated with a fluorescent ddNTP at its end, and this is detected by the sequencer using a laser beam. This way, the sequence of the amplified fragment is recreated and compiled as a chromatogram, which can then be used in succeeding analytic steps.

The diagram below provides a summary of the DNA sequencing procedure.



DNA Sequencing Procedure
Illustration by John Gregor A. Roño

References

- Karger, B.L., & Guttman, A. (2009). DNA sequencing by CE. *Electrophoresis* 30(S1): S196–S202.
- Sanger, F., Nicklen, S., & Coulson, A.R. (1977). DNA sequencing with chain-terminating inhibitors. *Proceedings of the National Academy of Sciences (U.S.)* 74(12): 5463–5467.

Sequence Assembly

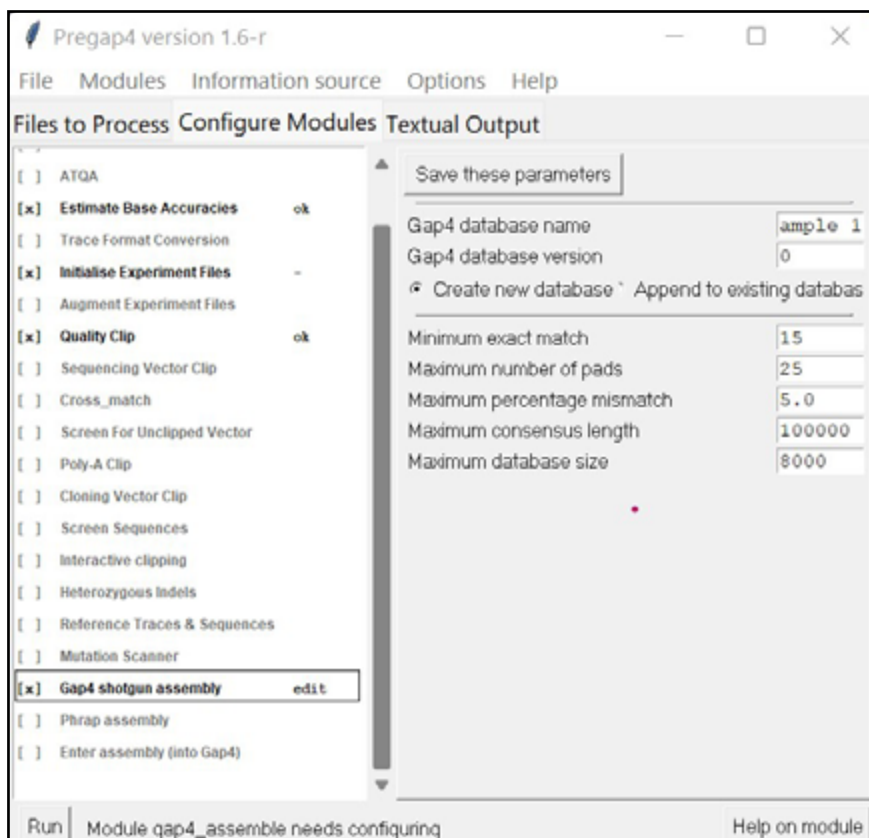
John Gregor A. Roño

Prior to sequence analyses, the raw DNA sequences must first be assembled using the STADEN package version 1.5.3 (Staden et al. 2000). Processing of sequences is necessary to check for ambiguous nucleotides or gaps. The forward and reverse strands that were amplified through the polymerase chain reaction (PCR) will be used to counter check each other and build a consensus sequence.

In the workshop for this manual, participants were provided with a set of raw sequences. Each sample is represented by four files, two for the forward sequence and two for the complementary or reverse sequence. For each sequence, there is an ab1 file, which represents the chromatogram generated from the direct sequencing step, and a seq file, which gives the nucleotide sequence based on the chromatogram. This manual uses the steps to process the sequences indicated in the STADEN package.

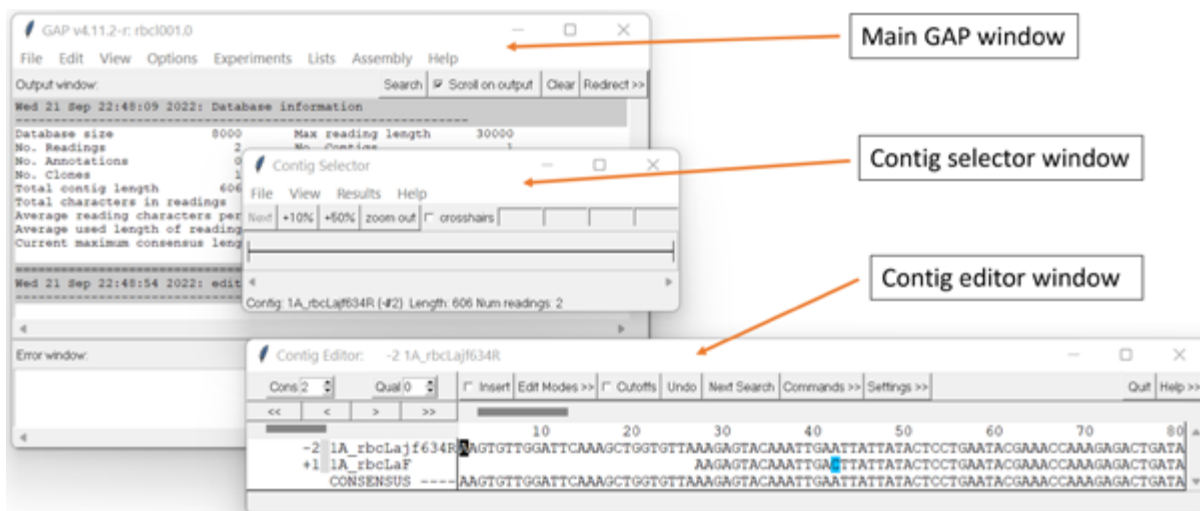
Procedure

1. Open the pregap4 program. Under **Files to process**, click **Add files**.
2. Go to the folder of a sample and select the ab1 files of the forward and reverse sequences.
3. Click **Configure Modules**.
4. Tick only the following: **Estimate Base Accuracies**, **Initialize Experiment Files**, **Quality Clip**, and **Gap4 shotgun assembly**.
5. Under **Gap4 shotgun assembly**, provide a name for the file on the space provided for **Gap4 database name**. Click **Create new database**.



Parameters to be ticked [x] under the Configure Modules tab.

6. Click **Run**. This will generate several files, including an aux file to view the consensus sequence based on the forward and reverse sequences.
7. Open the GAP program.
8. Click **File** and select **Open**.
9. Go to the folder of the sample you just worked on and select the .aux file.
10. A new Contig selector window will open. This window will tell you if you have successfully overlapped the forward and reverse sequences together. Note that pregap4 automatically selects one sequence and obtains its reverse complement so that it can be overlapped with the other sequence for easy comparison and generation of the consensus sequence. If you see only one line, then you have successfully overlapped the two sequences.
11. Go back to the main GAP window and click **Edit**, then select **Edit contig**. GAP will automatically select one sequence as the basis for the consensus sequence. Click **OK**.
12. A new Contig Editor will appear. It will show you the two sequences where they overlapped, as well as, the consensus sequences. Click on any nucleotide from the consensus sequence, and this will open a Trace Display window that shows the chromatograms of the two sequences and where they are aligned.
13. On the left side of the Contig Editor, inspect the primers (use the horizontal scroll bar). See if the forward primer is '+', and the reverse primer '-'. If yes, proceed to Step 15. If not, close the window and proceed to number Step 14.
14. On the GAP main window, click **Edit**, **Complement a contig**, and then **OK**. Then click **Edit** once again and then **Edit contig**. A window similar to Step 12 will appear, but this time, the signs beside the primer should have been corrected.
15. To allow you to edit the sequences, click **Edit Modes** from the **Contig Editor** window and select **Mode Set 2**.



Pertinent GAP windows for the analysis.

16. Click **Settings**. Make sure that only **Highlight disagreements**, **By background color** and **Store undo** are ticked. Click **Settings** again and then **Save settings**.
17. You should have with you the primer sequences that were used to amplify the DNA sequence you are processing. Do not include primer binding sites in the consensus sequence. To do this, go to the 5' end (leftmost end of the sequences) and locate the primer binding site by using the forward primer as reference. To hide the primer binding site, simultaneously type **Ctrl** and the right arrow on the keyboard and move the cursor until the entire primer binding region is hidden. Do the same thing for the 3' end (rightmost end of your sequences), but take note that you will have to get the reverse complement of your primer to locate the primer binding site.
18. Move across the entire alignment and check for any disagreements, which are depicted as shaded nucleotides. Correct the sequences using the chromatograms as a basis.
19. Once done, click **Quit** in the Contig Editor window and save all changes.
20. Click **File** in the main GAP window and select **Save consensus**, then **normal**. Give a filename for the consensus sequence in the Output file (e.g. filename.fas).
21. The consensus file is now ready for alignment and analyses.

Reference

Staden, R., Beal, K.F., & Bonfield, J.K. (2000). The Staden Package, 1998. *Methods in Molecular Biology* 132: 115–130.

Sequence Alignment

John Gregor A. Roño

Processed sequences can now be read by DNA sequence analysis software such as BioEdit version 7.0.9 alignment editor (Hall 1999). Positions in the alignment should be identified as unambiguous when there is absolutely no question over their positional homology. All other positions for which homology could not be ascertained should be excluded from subsequent analyses.

Procedure

1. Open the Bioedit program. Click **File** and go to the window containing the alignment file. Alternatively, you can also create a new alignment by clicking **Sequence** and selecting **New sequence**. You can paste your consensus sequence in the space provided and give a filename for the sequence.
2. To align the sequences, highlight all sequences by typing CTRL+A. Then click **Accessory Application** and select **ClustalW Multiple alignment**. Then click **Run ClustalW**. This will open a new window with all the sequences aligned. It may be necessary to align the sequences again by eye to further refine the alignment.

Reference

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

Poikilospermum suaveolens (Blume) Merr.
Photo by Edwino S. Fernando



The image features three spherical flower heads, likely from a species of Eucalyptus, in shades of pink and purple. They are arranged on a dark brown branch against a background of vibrant green leaves. The lighting is bright, creating a soft glow around the flowers.

PART 3

Sequence
Analysis and
Bioinformatics

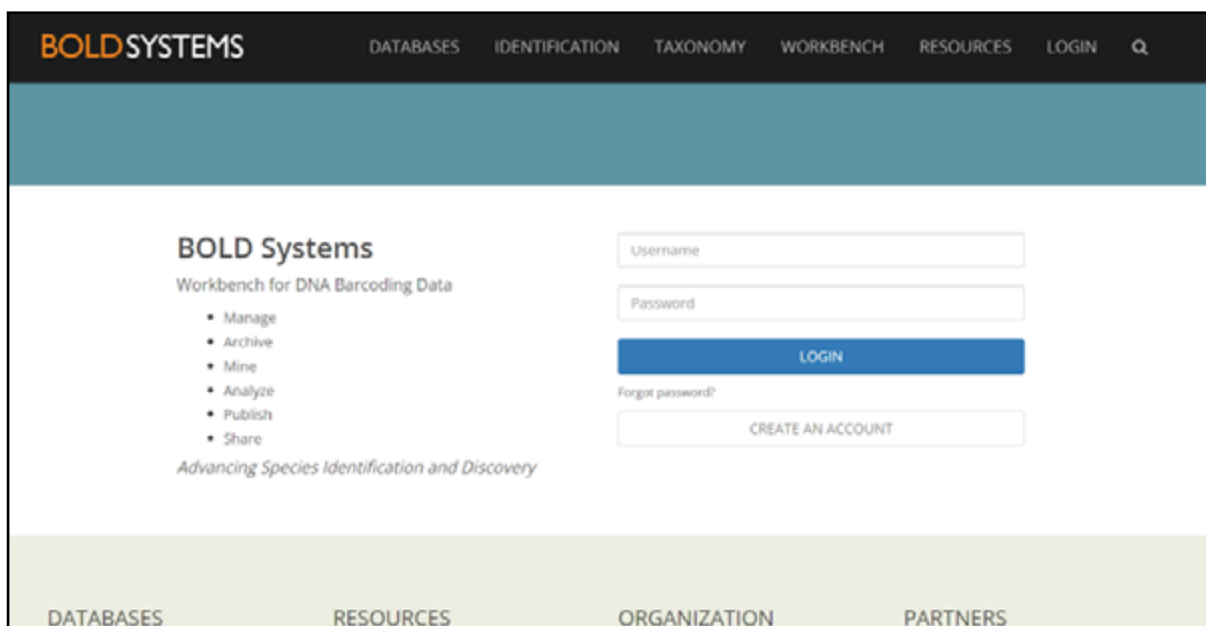
Registering New DNA Barcodes to a Database

Adrian U. Luczon

This section shows how to register new DNA barcodes to a publicly available database called the Barcode of Life Data System (BOLD). Currently in its fourth iteration (Ratnasingham & Hebert 2007), BOLD is an online repository for DNA barcodes of animals, fungi, and plants. Users of this platform can create projects and datasets that can be subjected to several built in bioinformatic analyses. You may contact BOLD support staff for your queries at support@boldsystems.org.

Registering an account

A user account is needed to deposit DNA barcodes, form projects, create datasets, and perform most bioinformatic analyses in BOLD. To register an account, follow the steps below.



The screenshot shows the BOLD Systems website interface. At the top, there is a navigation bar with the logo 'BOLD SYSTEMS' and menu items: DATABASES, IDENTIFICATION, TAXONOMY, WORKBENCH, RESOURCES, LOGIN, and a search icon. Below the navigation bar is a teal header section. The main content area is white and features the 'BOLD Systems' logo and tagline 'Workbench for DNA Barcoding Data'. A list of actions is provided: Manage, Archive, Mine, Analyze, Publish, and Share. Below this list is the tagline 'Advancing Species Identification and Discovery'. To the right of the text is a login form with fields for 'Username' and 'Password', a blue 'LOGIN' button, a 'Forgot password?' link, and a 'CREATE AN ACCOUNT' button. At the bottom of the page, there is a light green footer with navigation links: DATABASES, RESOURCES, ORGANIZATION, and PARTNERS.

Main page for logging in or creating an account in BOLD.

1. Go to www.boldsystems.org, and click on **Workbench** in the header. Under the login section click on **Create Account**.
2. In the next page, input your email address (preferably an official institution email), name, institutional affiliation, and chosen username and password. Your institution needs to be registered in BOLD. If another user has already registered it, the name of your institution will appear as you type it in the space provided. Simply select your institution as it appears. Otherwise, you may need to register your institution prior to continuing with registering your account.
3. Once you have typed your data, click on **Submit Request** and wait for an email from BOLD to confirm that your user data has been created.

Overview of your user console

You can now log in and access your user console once your account has been created. This page provides a quick overview of the user's data management activities. Users can also use this console to find created projects, view recent activities, and utilise search tools that can find publicly available projects and records within BOLD.

Users can also view and create new datasets right from this console. Datasets are barcode records pulled from one or several of your projects, as well as public records from other users.

On the quick access panel, several upload options are provided for specimen data, images, trace files, and sequences. Primer sequences registered by BOLD users can be accessed here whenever you need primer sequences. New primers can also be registered on this page. You can also access the publications section in this panel to register barcodes to Genbank for publication purposes. All sequence analysis tools and other functionalities of BOLD are found in this panel.

The screenshot shows the BOLD user console interface. A dark sidebar on the left contains the user's name 'Adrian H. Luczon' and a menu with options like 'Main Console', 'Projects', 'Checklists', 'Primers', and 'BOLD Main Menu'. The main content area is titled 'Welcome to BOLD Systems' and 'Home / BOLD Main Console'. It features several summary cards: 'Projects' with 32 items, 'Specimens' with 12362 records and 10931 barcodes, and an 'Uploads' section with buttons for Sequences, Traces, Images, Primers, Publication, and Checklist. Below these are two tables: 'Your Datasets: 7' and 'Recently Accessed'. The 'Your Datasets' table has columns for Code, Title, and Specimens. The 'Recently Accessed' table has columns for Code, Title, Specimens, and Accessed. Red arrows from the text above point to the 'Quick Access Panel' (the sidebar), 'Owned Projects and Assets' (the Projects and Specimens cards), 'Search Tool' (the search bar at the top), and 'Recent Activities' (the Recently Accessed table).

The BOLD user console

Creating a project

A project must be created before uploading DNA barcodes to the database. Projects are used in BOLD to store similar records, and allow users to handle the sharing, permissions, and the publication of groups of records. Projects can be created based on research topics, sampling locations, taxonomic groups of interest, and others. Records can be moved between projects as needed.

Procedure

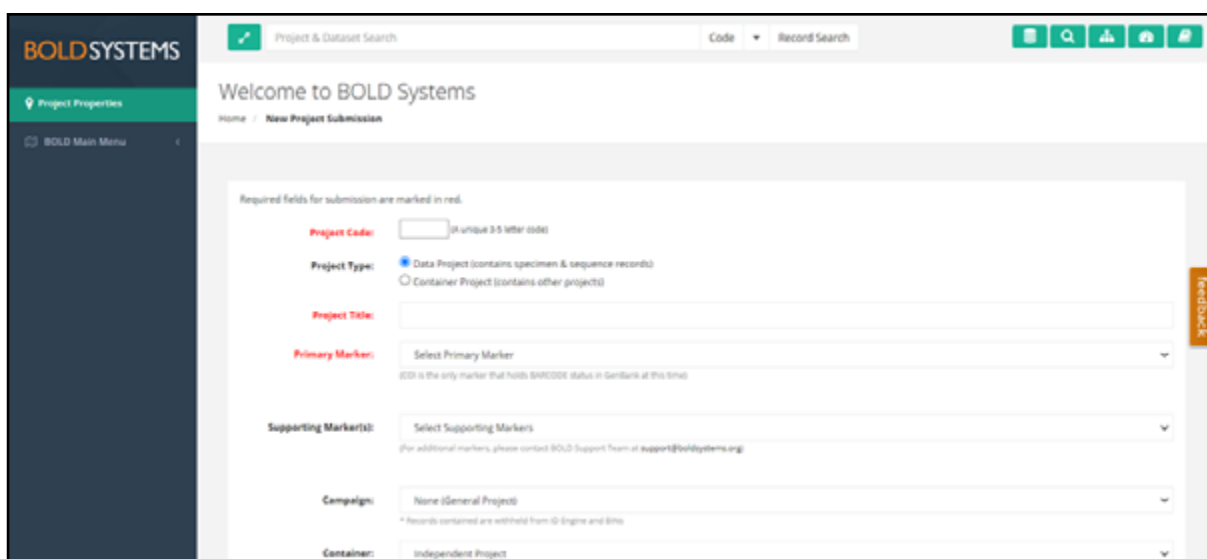
1. Log into BOLD. From the quick access panel on the left, click on **Projects**, and then **+ New Project**.
2. In the new page, fill in the following required fields:

Project code: A unique 3–5 letter code you will give for your project. BOLD will notify you if the code you typed has already been taken by another project. In such a case, you need to provide a different set of code.

Project title: Title for the project, preferably describing a central theme for your barcode records.

Primary marker: The primary genetic marker you will use for the barcode records. Click on the drop-down menu and choose the marker that you used for your barcoded specimen. *matK* and *rbcL* are markers for plants that are supported by BOLD. You may also provide supporting markers by clicking the Supporting marker(s) drop-down menu.

Project description: A more detailed description of the project.

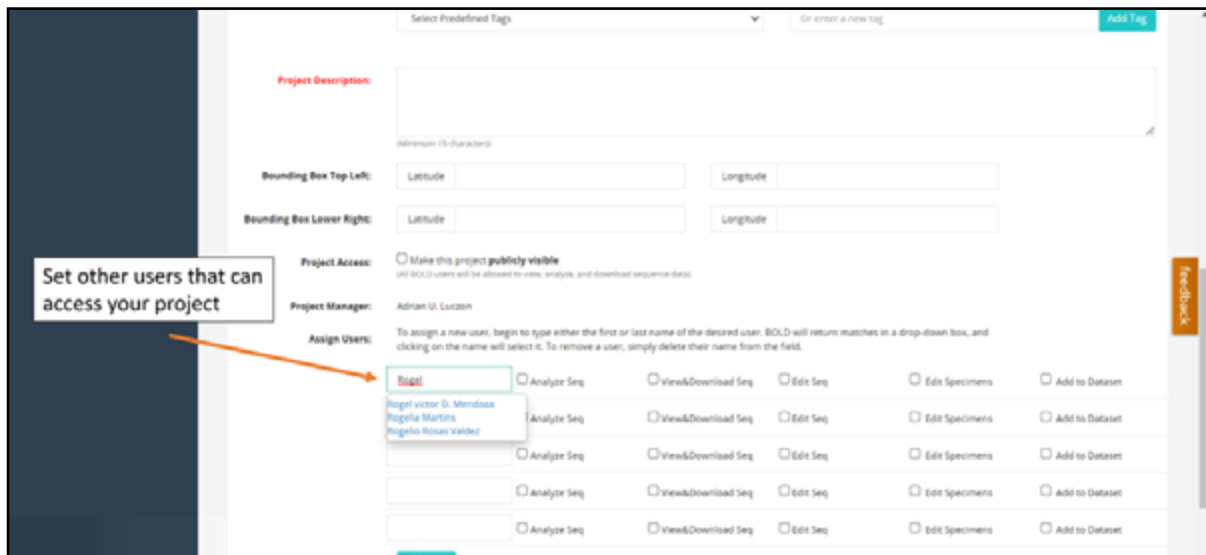


The screenshot shows the 'New Project Submission' page in the BOLD Systems interface. The page has a dark blue sidebar on the left with 'Project Properties' and 'BOLD Main Menu' options. The main content area is white and contains a form with the following fields:

- Project Code:** A text input field with a red asterisk and the note '(A unique 3-5 letter code)'. The field is currently empty.
- Project Type:** Two radio button options: 'Data Project (contains specimen & sequence records)' (selected) and 'Container Project (contains other projects)'.
- Project Title:** A text input field with a red asterisk, currently empty.
- Primary Marker:** A dropdown menu with a red asterisk and the text 'Select Primary Marker'. Below it, a note states: 'rbcL is the only marker that holds BOLD/DOI status in GenBank at this time!'. The dropdown is currently set to 'None'.
- Supporting Marker(s):** A dropdown menu with a red asterisk and the text 'Select Supporting Marker(s)'. Below it, a note states: '(For additional markers, please contact BOLD Support Team at support@boldsystems.org)'. The dropdown is currently set to 'None'.
- Campaign:** A dropdown menu with a red asterisk and the text 'None (General Project)'. Below it, a note states: '* Records contained are withheld from ID Engine and IDs'. The dropdown is currently set to 'None'.
- Container:** A dropdown menu with a red asterisk and the text 'Independent Project'. The dropdown is currently set to 'Independent Project'.

BOLD page for creating a new project.

- At the lower section of the project creation page, you can specify registered users that can access the project and set specific activities that they can perform. Other optional features can be set in the project. Once you have finished typing in the details, click **Save**. Your project is now created.



Adding registered BOLD users that can access your project.

- By default, you are the Project Manager of this project. The project manager can be changed, but you will have to contact BOLD support staff. The project manager can modify the project properties and user permissions at any time by clicking on **Projects** from the quick access panel. Click on **View All Projects** and then click on the project of interest. On the quick access panel once again, click on **Project Options** and then **Modify Project Properties**. From this page, you will notice that you cannot change the **Project Code** and **Project Type**.

Uploading specimen data

Once a project has been created, you may now start populating the project with specimen data. You may do this even before DNA sequences have been generated from the samples. Users may choose to upload specimen data as a batch using an MS Excel file and then sending it through a portal in BOLD. Using this method, BOLD staff will input all entries to your project, which may take a few days. The steps below show how to upload single records manually to the system through the **Specimen Data Upload** option available in the **Project Console**. This is the fastest way to add an individual record to a project. Project managers and users granted access to edit specimens are allowed to upload specimen data.

Procedure

- Go to your project, by following the steps in the previous section.
- Click on **Uploads**, and then **Specimen Data**.

3. A new page will open containing a form where the specimen data can be entered. The **Initiate Batch Submission** button on the page is the portal for submission of the previously mentioned MS Excel file.
4. The following data are the minimum required entries for each specimen data:
Sample ID – this is a unique code for the sample being sequenced. This field needs to be unique relative to all records in BOLD. You will be notified if the Sample ID has already been used.

Field ID – this is a specimen's unique code set in the field.

Museum ID – this is a specimen's unique code assigned by a formal collection such as a museum or herbarium. The Field ID and Museum ID are required fields. If these codes are not present, you may simply use the Sample ID.

Institution Storing – this is the institution storage of the voucher specimen. The institution must be registered. If another user has already registered it, the name of the institution will appear as you type in the space provided. Simply select the institution as it appears. Otherwise, you may need to register the institution. Private collections are also allowed.

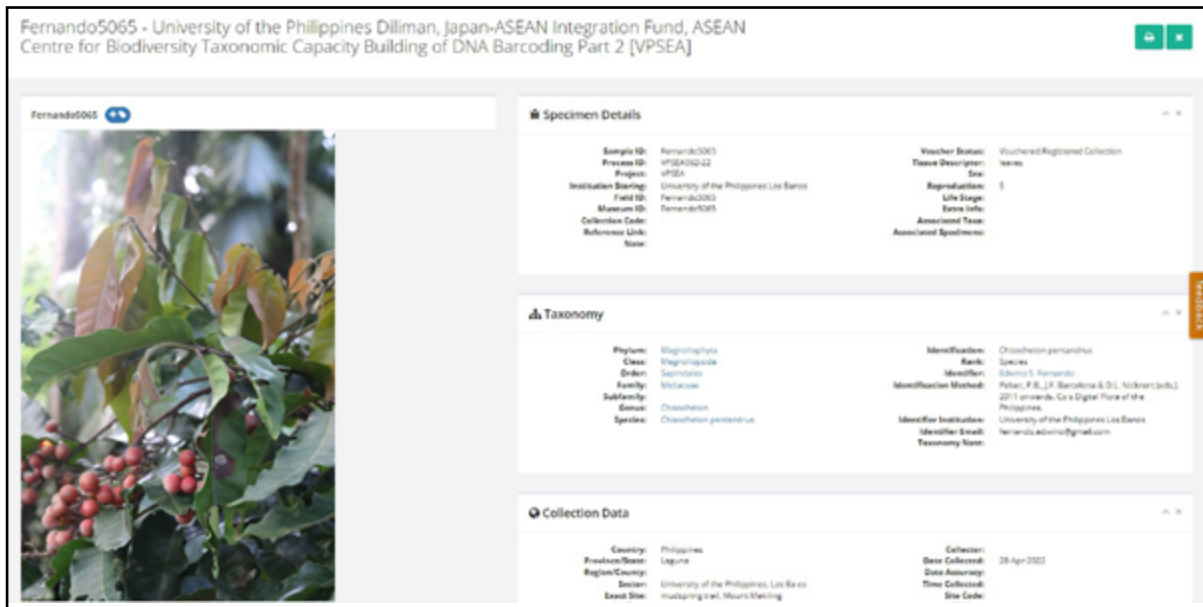
Phylum – minimum level of species taxonomy required for your specimen. Having a species level identification is preferred for it to be useful for the DNA barcoding community.

Country – minimum level of geographic specificity where the sample was collected. Country inputs should be based on the list found in the International Organization for Standardization (ISO).

The screenshot shows the 'Specimen Data Submission' page in the BOLD system. The page has a dark blue sidebar with a white 'B' logo. The main content area is white with a light blue header. The title 'Specimen Data Submission' is in the top left. Below the title is a green button labeled 'Initiate Batch Submission'. Underneath is a section titled 'Specimen Identifiers' with several input fields: 'Sample ID:', 'Field ID:', 'Museum ID:', 'Collection Code:', and 'Institution Storing:'. The 'Institution Storing' field is highlighted with a red border. Below the 'Institution Storing' field is a small note: 'To choose the institution storing, type in the name and select it from the drop-down list of matches.'

BOLD page for adding specimen data

- Other details about the specimen data such as life stage, collection date, collector, GPS data, and others can be indicated and may be updated later. Click on **Submit** when finished.
- Your first specimen data should now be visible in your project. To view this record, click **View All Records** from the quick access panel.
- Repeat the Steps to continue uploading specimen data of your other samples.



Example of a BOLD record with specimen data and uploaded image

Uploading specimen images

You may upload images as a batch file like the batch submission of specimen data. The steps below show how to upload the image from the specimen page of your record.

Procedure

- Go to your project and click on **View All Records** in the quick access panel.
- The sample ID of each specimen data is a clickable link. Click on the sample ID where the images will be uploaded.
- Click **Edit Specimen** in the new page.
- Click the **Upload Image** button.

5. You will be taken to a page where you will upload the image and specify some details from it. The following are required fields:

Image file - Click on **Choose File**, click the photo you wish to upload. You can only upload one photo at a time.

Original specimen – Indicate if the image was taken from the specimen where the DNA sequence originated.

Orientation/View Metadata - Orientation of the specimen in the image (e.g. Dorsal, Ventral, Proximal, and others)

License - Select from a drop-down list

License institution – You may choose your own institution or register a different institution.

6. Other details of the image can be entered but are optional.
7. Click **Submit** once done. The image should now be visible on the specimen page of that record. Multiple images can be uploaded to a record, so repeat the process as necessary.

Uploading sequences

The sequences are submitted as a FASTA format, which is a text document containing a list of all the DNA sequence data. The Sample IDs should be used in naming the sequences.

Procedure

1. Open an alignment file containing the DNA sequences you generated using BioEdit.
2. You need to change the sequence names of the sequences in the alignment file to match the sample IDs in your BOLD project. Double click on the sequence name in the alignment file to change the name. To view the list of Sample IDs in your project, go to your project once again and click **View All Records**.
3. Once you have finished changing the sequence names, save the file. You may want to save the file with a different file name.
4. Go back to your project in BOLD and click **Uploads**. Click on **Sequences**. You will be taken to a new page where you can paste the DNA sequences.
5. On Select ID, choose **Sample ID**. Under **Markers**, select the appropriate marker used as the DNA barcode (e.g. *rbcL*, *matk*). Under **Run** site, you may need to add an institution if it's not registered in BOLD. The Run site is the institution where the sequences were generated.

6. Open your alignment file using a text program such as Notepad. Each sequence begins with a '>', followed by the Sample ID. Copy all the sequences and paste it on the space below Paste sequences in FASTA format.
7. Click **Submit**. The DNA sequences should now be visible in the Sequence page of your specimen.

Uploading trace files

The Single Trace Upload tool can be accessed via the Sequence Page in the project's record list. A maximum of four electropherograms (usually .ab1 files) and four optional Phred/score files (usually .phred files) per loci can be uploaded at a time. However, they must be from the same sequencing direction.

Procedure

1. Go to your project. Click on **View All Records**.
2. Each of the records will have a **Process ID** under the **Sequence Page** column. The Process ID is a link to the sequence page of that record. Click on the **Process ID** of the specimen you wish to upload the traces.
3. From the new window, click on **Upload Traces**.
4. You will be sent to a page where you can upload the trace files and indicate the primers used during PCR and DNA sequencing.
5. Select the Marker used for the DNA sequence.
6. Choose the sequencing lab. You may need to register the lab if it is not in the record of BOLD.
7. Indicate the Forward and Reverse primers used during PCR.
8. Indicate the sequencing direction of the trace file.
9. Select the relevant .ab1 and/or .phd1 from your computer.
10. Click Upload. Repeat these Steps for the trace files that are in the other direction. Removing trace files from the record of your specimens can only be done by contacting BOLD support.
11. The sequences and trace files should now be visible in the sequence page of your record.

Upload Traces
✕

Marker: COI-5P ▼

Sequencing Lab:

Select My Institution
Or type in the name of your institution and select it from the drop-down list of matches.

PCR Forward Primer: 1709Fg ▼

PCR Reverse Primer: 2191R ▼

Sequencing Primer Direction: Forward Reverse

Sequencing Primer: Select Sequencing Primer ▼

Upload up to 4 trace files and phred files. (Phred files are not required for trace upload)

<p>Trace 1: Choose File No file chosen</p> <p>Trace 2: Choose File No file chosen</p> <p>Trace 3: Choose File No file chosen</p> <p>Trace 4: Choose File No file chosen</p>	<p>Phred 1: Choose File No file chosen</p> <p>Phred 2: Choose File No file chosen</p> <p>Phred 3: Choose File No file chosen</p> <p>Phred 4: Choose File No file chosen</p>
---	---

Upload
Close

BOLD page for uploading trace files

Reference

Ratnasingham, S. & Hebert, P.D. (2007). BOLD: The Barcode of Life Data System (<http://www.barcodinglife.org>). *Molecular Ecology Notes* 7(3): 355–364.

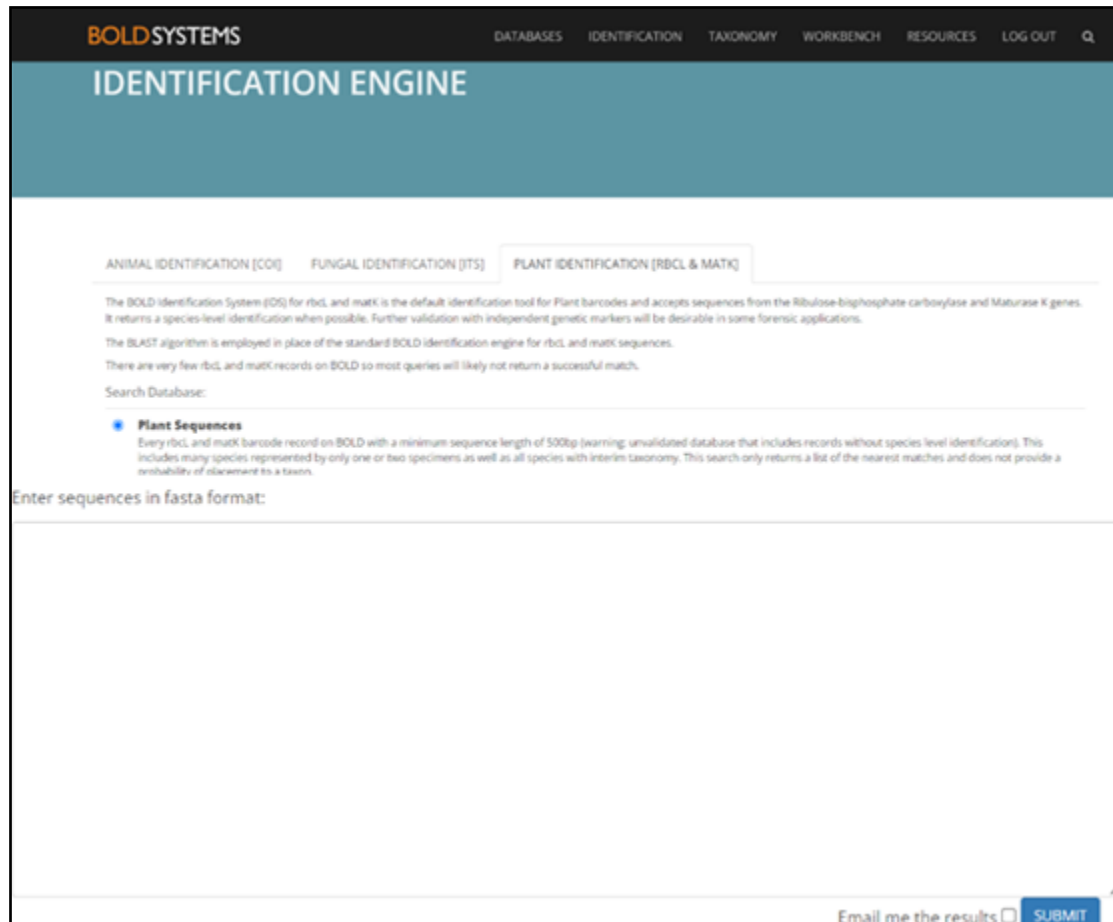
Bioinformatics Tools for Species Identification

Adrian U. Luczon

Bioinformatics is a field of science that applies tools of computational analysis to biological data. This manual uses the Identification Engine of Barcode of Life Data System (BOLD) and Basic Local Alignment Search Tool (BLAST) analysis from Genbank.

BOLD identification engine

The records within BOLD can facilitate the identification of unknown sequences. The algorithm searches all records from public and private projects to obtain the closest match for your unknown sequence. However, the sequences from private projects will not be visible.

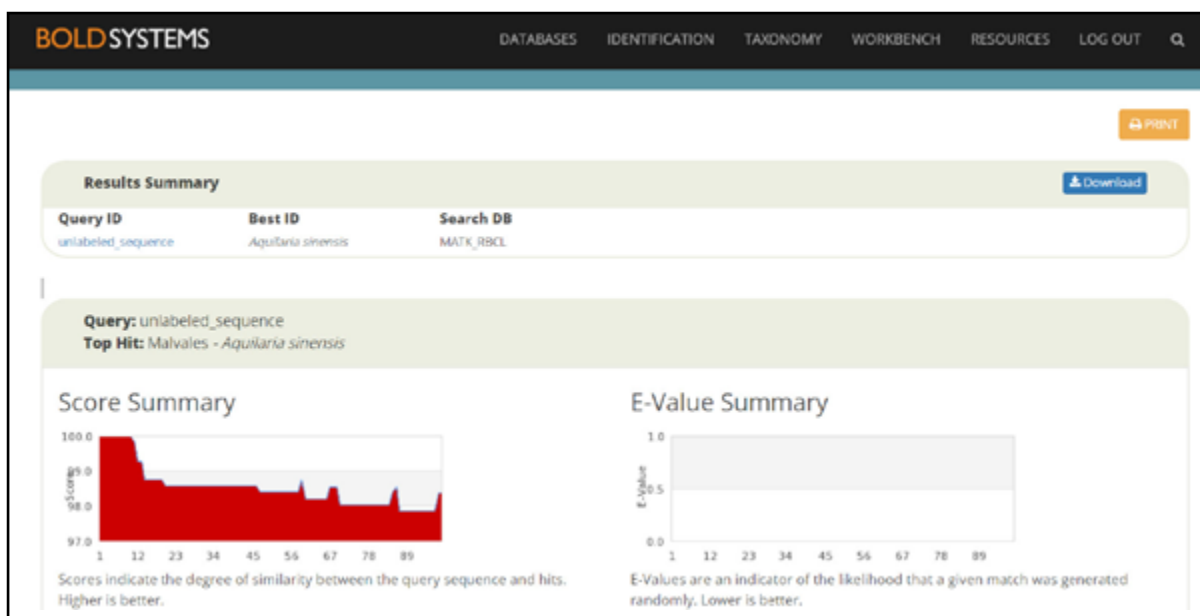


The screenshot shows the BOLD Identification Engine web interface. At the top, there is a navigation bar with the BOLD SYSTEMS logo and links for DATABASES, IDENTIFICATION, TAXONOMY, WORKBENCH, RESOURCES, LOG OUT, and a search icon. Below the navigation bar is a teal header with the text "IDENTIFICATION ENGINE". The main content area has three tabs: ANIMAL IDENTIFICATION [COI], FUNGAL IDENTIFICATION [ITS], and PLANT IDENTIFICATION [RBCL & MATK]. The PLANT IDENTIFICATION tab is selected. Below the tabs, there is a paragraph of text explaining the BOLD Identification System (ID) for *rbcl* and *matK*, mentioning the BLAST algorithm and the search database. A "Search Database:" section is followed by a radio button for "Plant Sequences" which is selected. Below this, there is a warning about the search results. A large text input field is labeled "Enter sequences in fasta format:". At the bottom right, there is a checkbox for "Email me the results" and a "SUBMIT" button.

BOLD Identification engine

Procedure

1. From the main page of BOLD, click on **Identification**. For plant specimens, click on the **Plant Identification** (*rbcl* and *matK*) tab.
2. Below the page, a space is available to paste your fasta formatted sequences.
3. Paste the sequences. Only *rbcl* and *matK* markers can be run on the identification engine of BOLD at this time.
4. Click **Submit**. If you are using the ID engine for many sequences, the analysis may take time. Click on the **Email me the results** before clicking **Submit**. A link to the results will be emailed once the analysis is done.
5. From the results page, the **Results Summary** section will show the Best ID of your unknown sequence.



Sample result from the BOLD ID engine

- Scrolling down the page will take you to the list of the top 100 matches of your sequence in the records of BOLD. The *rbcL* and *matK* alone cannot discriminate well between species. Results may be inaccurate, especially if there are different species that are similar to your unknown sequence. In such cases, a concatenated sequence of *rbcL*, *matK*, and other markers may be analysed using a phylogenetic tree (see succeeding sections.)

Genbank

The National Center for Biotechnology Information (NCBI) website (<http://www.ncbi.nlm.nih.gov/>) has several bioinformatics tools for data mining and sequence analysis. These include BLAST, which searches for regions of local similarity between sequences.

The different BLAST programmes are given in Table 2.

Table 2. BLAST Programmes

Programme	Use
blastn (nucleotide blast)	Compares a nucleotide query sequence against a nucleotide sequence database
blastp (protein blast)	Compares an amino acid query sequence against a protein sequence database
blastx	Compares a nucleotide query sequence conceptually translated into 6 reading frames against a protein sequence database
tblastn	Compares a protein query sequence against a nucleotide database translated into 6 possible reading frames
tblastx	Compares a nucleotide query sequence translated into 6 possible reading frames against a nucleotide database translated into 6 possible reading frames

This manual uses **blastn** to identify your unknown sequence.

Procedure

1. Go to the NCBI homepage (<http://www.ncbi.nlm.nih.gov/>) and click **BLAST** in the menu bar on the right. Click on **Nucleotide BLAST**.
2. Paste your fasta formatted sequence onto the text box. You may use the sequences you uploaded in BOLD for this activity.
3. Under the **Choose Search Set** menu, click **Standard databases (nr etc)**.
4. Under **Program Selection**, click **Highly similar sequences (megablast)**. Then click **BLAST**.
5. Examine the BLAST report window. The BLAST result page contains the **Summary** on the top-left corner, **Descriptions** tab, **Graphic Summary** tab, and **Alignments** tab. The **Summary** contains information about the searched database and the query sequence. If you run multiple sequences for BLAST analysis, each of the BLAST results for each unknown sequence can be accessed by clicking the drop-down menu beside Results.

The screenshot shows the NCBI Nucleotide BLAST web interface. At the top, it says "BLAST® » blastn suite" and "Standard Nucleotide BLAST". Below this, there are tabs for "blastn", "blastp", "blastx", "tblastn", and "tblastx". The "blastn" tab is selected. The main section is titled "Enter Query Sequence" and contains a text area with a FASTA sequence: ">Specimen 1\nGTGTTAAAGAGTATAAATTGACTTATTATACTCCTGAATATGAAACCAAGAT\nACTGATATCTTGGCAGC\nATCCGAGTAACTCCTCAACCTGGAGTCCGCCTGAGGAMGCAGGGGCT". To the right of the text area are fields for "Query subrange" (From and To). Below the text area are fields for "Or, upload file" (Choose File) and "Job Title" (Specimen 1). The "Choose Search Set" section has radio buttons for "Standard databases (nr etc.)", "rRNA/ITS databases", "Genomic + transcript databases", and "Betacoronavirus". A dropdown menu shows "Nucleotide collection (nr/nt)". There is also an "Organism" field with an "Add organism" button.

Webpage of nucleotide BLAST.

The **Descriptions** tab gives a list of the sequences in Genbank that are most similar to your query sequence. By default, the algorithm arranges the list based on the lowest E-value. This value gives an indication of statistical significance. It is a parameter which describes the number of hits one can expect to see simply by chance when searching a database of a particular size. We will use this parameter for all our analysis.

In the **Graphical summary** tab, the items in the **Descriptions** tab are represented as coloured bars. The query sequence is represented by the teal-coloured bar (topmost). Database hits are shown as bars below the query. As with the **Descriptions** tab, the bars are arranged based on the lowest E-value by default.

The **Alignments** tab provides the sequence alignment between the query and database sequences.

6. Return to the **Descriptions** tab and determine the closest match of your unknown species (topmost record from the list).
7. As mentioned previously, individual results from *matK* and *rbcL* sequences could be inaccurate. In such cases, a concatenated sequence of *rbcL*, *matK*, and other markers may be analysed using a phylogenetic tree (see succeeding sections).
8. In the **Descriptions** tab, the top 100 sequence matches can be downloaded by clicking **Select All**. Click **Download** and choose **FASTA** aligned sequences. Give the alignment a file name. Change the file extension to 'fas,' instead of 'txt' and save. These sequences can now be used for the succeeding section.

Phylogenetic Tree Construction

Adrian U. Luczon

This manual follows the Neighbor Joining (NJ) method to construct a phylogenetic tree. Phylogenetic trees are graphical representations of the evolutionary relationships among taxa derived from morphological or molecular datasets. The neighbor-joining method is a distance algorithmic method that uses a modified distance matrix that adjusts the separation of each pair of sequences based on the pair's average divergence with all the other sequences. This method identifies the shortest tree by sequentially finding neighbors that minimise the total length of the tree.

At this stage, you will use your generated DNA sequences as well as DNA sequences that are available from the databases (Genbank or Biodiversity of Life Data System). The sequences from the database may include all available DNA sequences belonging to the taxonomic family of your specimens and/or sequences closest to your specimen based on the BLAST results of Genbank and Species ID from BOLD. These downloaded sequences will serve as your reference sequences (DNA sequence of a specimen with known species). For this manual, the software MEGA version XI (<http://www.megasoftware.net/>) is used to generate an NJ tree.

Concatenating the DNA sequences

If multiple markers were sequenced for each specimen (e.g. *rbcL*, *matK*, and ITS), we must first concatenate these sequences or join the sequences from end to end. Likewise, the sequences downloaded from the database should also have the same markers. Ideally, you would download sequences that were generated from the same sample or specimen.

Procedure

1. Place all alignment files in one folder.

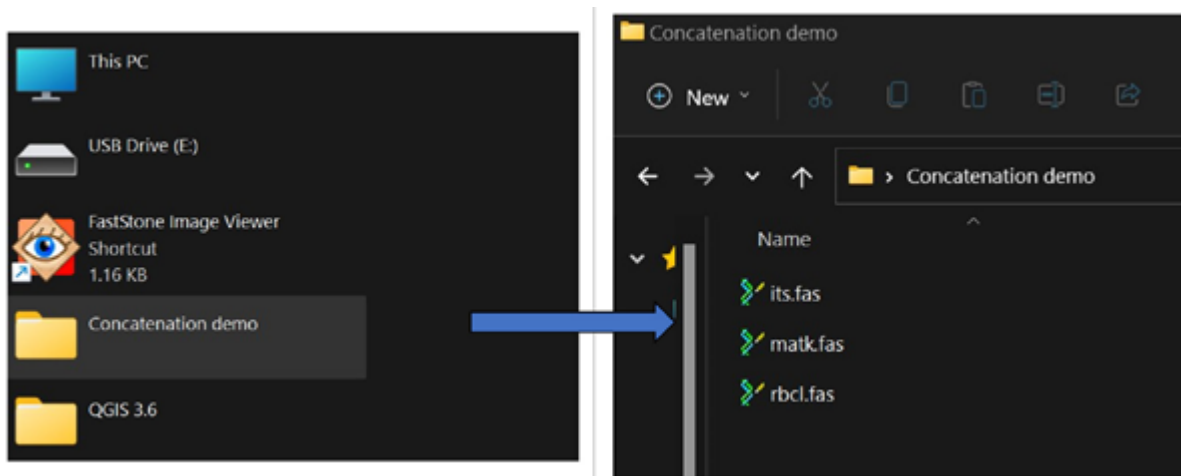
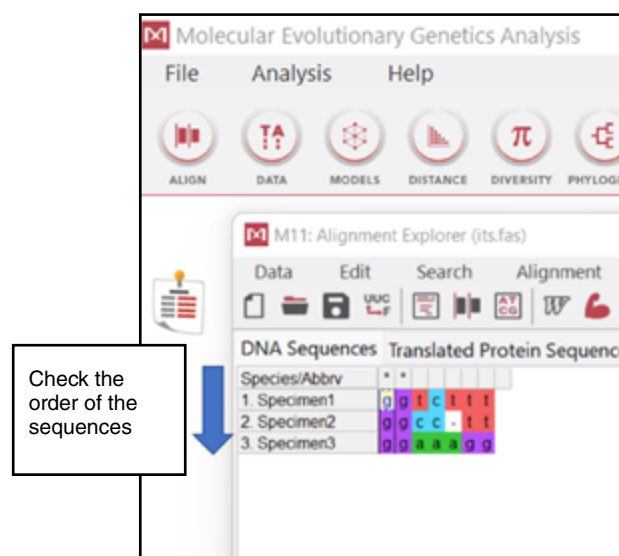


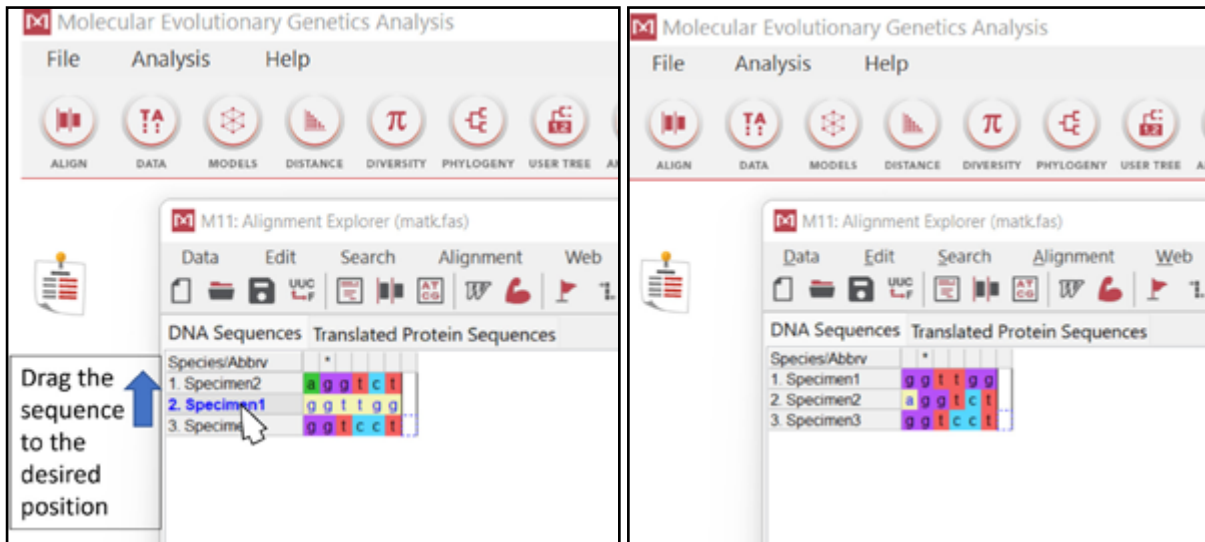
Image showing alignment files inside one folder

2. Make sure that sequences within each alignment file are listed in the same order and have the same names. Open MEGA XI. Open an alignment file by clicking **File**, then **Open A File/Session** and click on any of the alignment files. Click **Align** on the next window that will appear. A new window will appear where you can examine the order and names of the sequences.



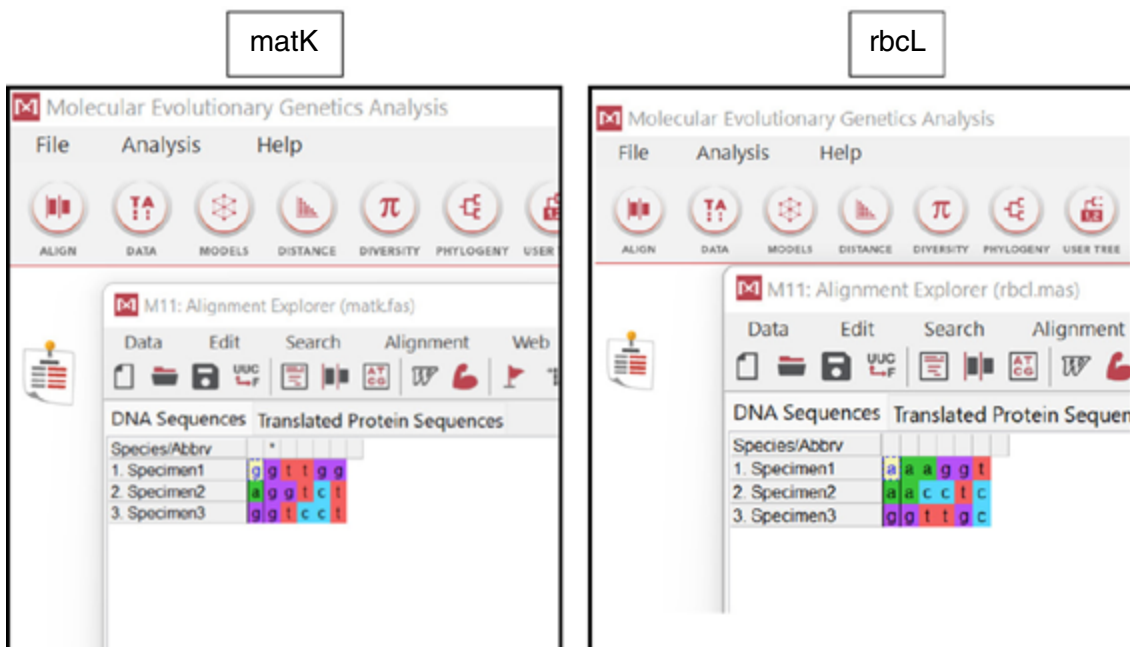
MEGA XI window showing the arrangement of the specimens and DNA sequences

- Open the other alignment files using the procedure in Step 2. Check if the order of the sequences and the sequence names are the same with the first file you opened. If not, simply click any of the sequences you wish to move and drag it to the correct placement. To change the name of a sequence, simply double click the name you wish to change and then edit. After editing the sequences, click **Data**, **Export alignment**, then **FASTA Format**. Click **Save**.



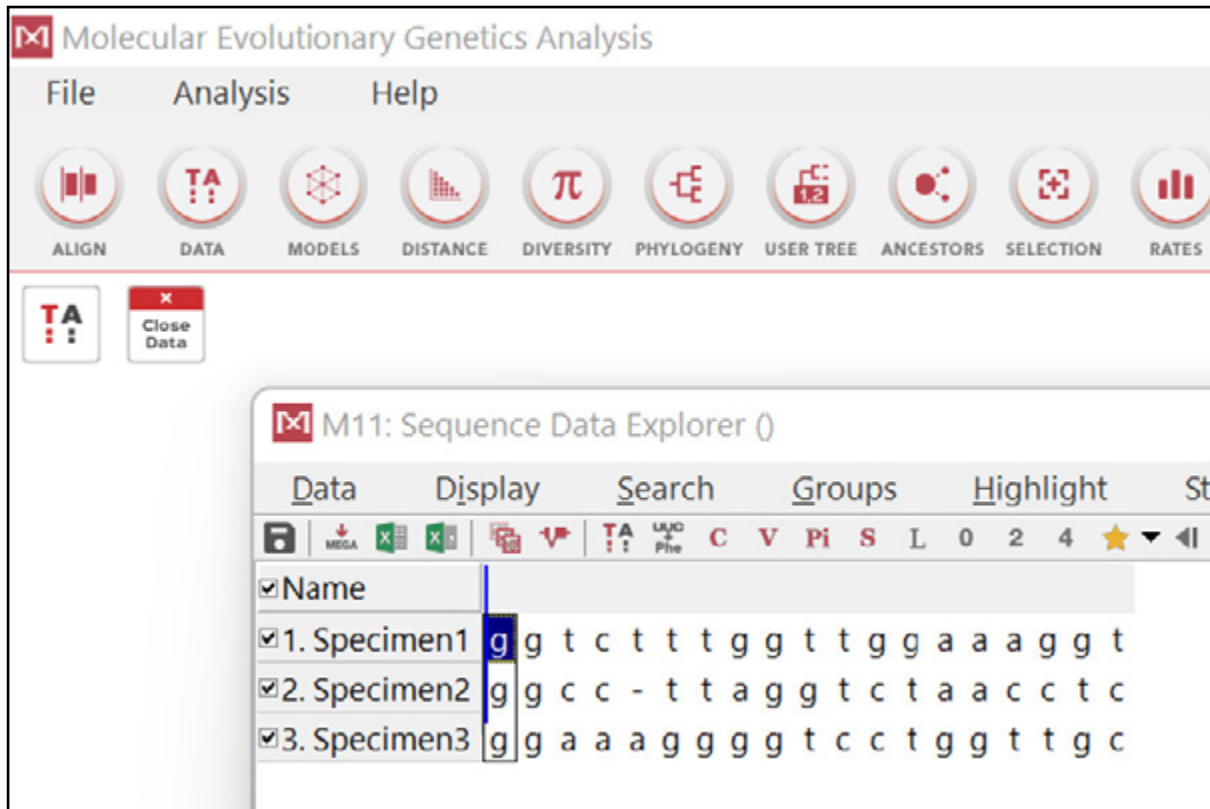
MEGA XI window showing how to rearrange the specimens

- All alignment files should now have the same order and name of sequences.



MEGA XI window showing that *matK* and *rbcL* specimens have the same names and arrangement

- Go back to the main window of MEGA XI. Click on **Data**, then **Concatenate Sequence Alignments**. Select the folder where the alignment files are located (the folder should only contain the alignment files to be concatenated). Click **OK** in the next window. Click on the '**TA**' box to view the concatenated sequences. The three alignment files should now be concatenated.



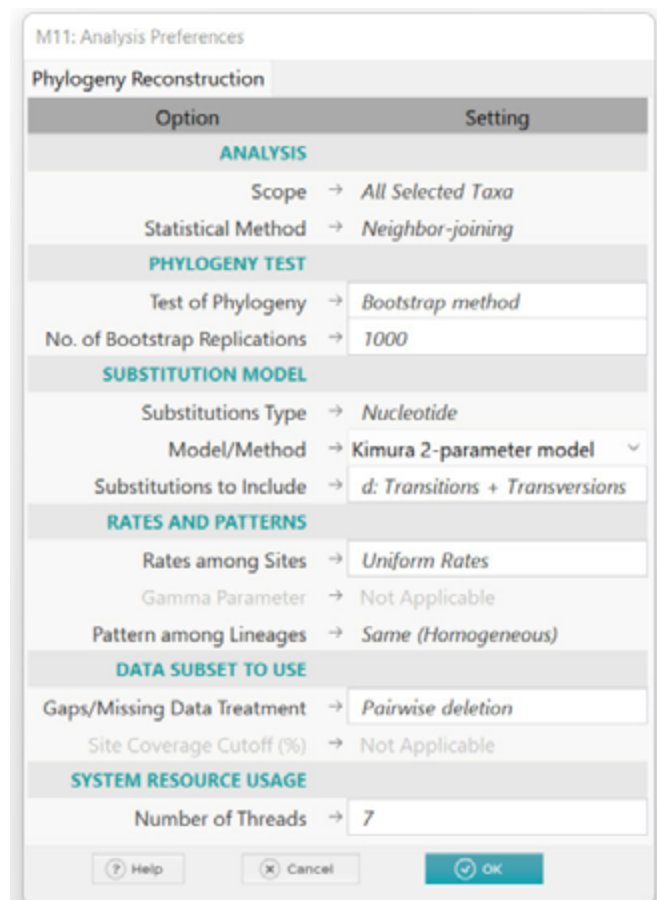
MEGA XI window showing a concatenated sequence

- Save concatenated sequences by clicking **Data**, then **Export Data**. In the new window, change the format to FASTA, then click **OK**. Save the file of the concatenated sequences to your desired location.

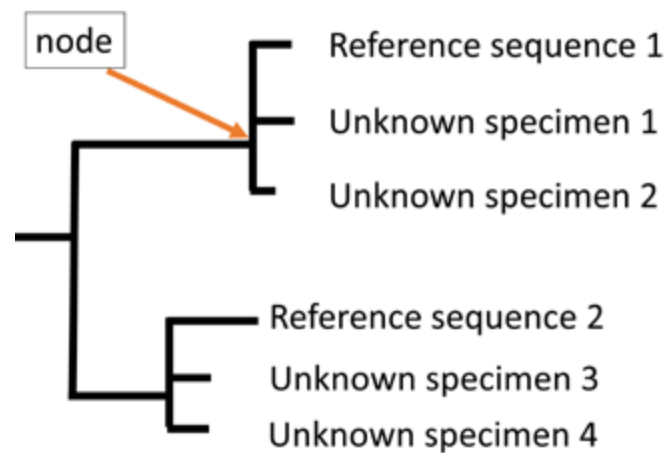
Phylogenetic tree construction

The instructions below are procedures for constructing a neighbor-joining tree.

1. Open MEGA XI.
2. Click **File, Open A File/Session**, then open the concatenated sequence file.
3. A new window will appear asking if you want to analyse or align the sequences. Since the sequences have already been aligned, click **Analyze**. In the new window, click on **Nucleotide Sequences**, then click **OK**. The program will ask if you are using a protein coding DNA sequence. Since the concatenated data is a mix of different markers, click **No**.
4. On the main window, click **Phylogeny, Construct/Test Neighbor-Joining Tree**, then click **Yes**. This will bring you to a new window where parameters for the NJ tree can be set.
5. In the window, under **Test of Phylogeny**, click the **Bootstrap** method. Set the number of bootstrap replications to 1000. The bootstrap method is a probability-like tool that will give confidence to each of the branches. Under model, set it to Kimura 2-parameter. Under **Rates among Sites**, change it to **Uniform rates**. Click **Compute**. The programme will now start constructing the tree.



MEGA XI window for setting the parameters of a neighbor-joining tree



Sample neighbor-joining tree

6. A new window will appear showing the constructed NJ tree. Root the tree on an appropriate outgroup taxon (the taxon that is most divergent or most different from all the other taxa). Taxa in this dataset are shown at the ends of the branches. Bootstrap supports are placed at the nodes.
7. Examine the tree. If the goal of the construction of the phylogenetic tree was to identify unknown species, then look closely at the position of the unknown species relative to the reference sequences included in the alignment. If the unknown species shares a node with a reference sequence, then most likely, the unknown species shares the same species name as the reference sequence. In the case below, unknown specimens 1 and 2 might be the same species as reference sequence 1, while unknown specimens 3 and 4 might be the same species as reference sequence 2.
8. If your unknown sequences do not share a node with any of the reference sequences, then it might be possible that your unknown sequence is a cryptic species or a new species record. In such a case, the tree should be examined closely and possibly employ the help from an expert.



Pothos scandens L.
Photo by Edwino S. Fernando



PART 4



Herbarium
Management

Management of Plant Specimen Collections

Edwino S. Fernando



Showing some of the procedures in the herbarium.



Photos by Patricia S. Leuterio

The science of taxonomy provides the basic knowledge underpinning plant biodiversity conservation and sustainable use. It provides the core reference system and knowledge base on which all discussion of plant biodiversity hinges. This core reference system is built on what plant taxonomists do:

- Plant identification (How can plants be recognised? What characters are useful to identify plants?)
- Nomenclature (What should plants be correctly called? How are these names determined?)
- Phylogeny and classification (What are their closest relatives? Are there any other plants likely to have similar properties? How is a genus or family best circumscribed?)
- Geographic distribution (Where do they grow?)
- Ecology (What kinds of habitat do they prefer?)
- Economic botany (What are their useful properties?)

The resolution of answers to these questions requires extensive research and examination of the plants themselves – living and dead – and all the information associated with them. Thus, the herbarium, in which these dead plants or parts are stored, is an essential working tool for the plant taxonomist. Initially referred to as *hortus siccus* (dried garden) (Rouhan & Gaudeul 2014), the herbarium may be defined as a collection of dried plant specimens, preserved, stored, catalogued, and arranged in an accessible system. The term ‘herbarium’ was first used by Joseph Pitton de Tournefort in 1700. It was subsequently picked up by Carl Linnaeus and has been used in this context ever since.

The Purpose and Importance of an Herbarium

The herbarium has long been traditionally used for research on the taxonomy of plants, but has also been explored by researchers in other plant-related sciences (after Bridson & Forman 1989):

As storage of voucher specimens used in research. Voucher specimens serve as material evidence for the plant and are indispensable for maintaining confidence in data linked to scientific names (owing to the changing circumscription of species as knowledge of flora advances) (Suarez & Tsutsui 2004; Funk et al. 2005); they are also permanent records of plants used in research, e.g. ecology, anatomy, palynology, phytochemistry, or DNA studies (Eisenman et al. 2012; Culley 2013). This requires adequate management for the curation of specimens and a form of indexing or accessioning and databasing.

A means of identification and arbiter of correct names. Unnamed plants can be matched with correctly named specimens in the herbarium. This also requires that the collections and associated database are regularly updated for changes in nomenclature in line with current taxonomic revisionary or monographic work.

A comprehensive databank and potential use for additional or further research. Ideally, the collections have been accessioned, with the associated data from the field and herbarium labels entered into a database and the actual specimens digitally photographed. The specimens themselves can also be used for further research in genetics, ecology, and environmental studies - for example, flowering phenology or stomatal leaf density in relation to climate change (Lavoie & Lachance 2006; Culley 2013; Davis et al. 2015; Heberling et al. 2019). Some old specimens held in herbaria may sometimes be found to represent new species. A study suggests that, of the estimated 70,000 species of plants still to be described, more than half already have been collected and are stored in herbaria (Funk 2004; Bebbler et al. 2010).

Types of Herbaria and their Function

Different types of herbaria may be defined based on the kinds of specimens they house (after Victor et al. 2004). The actual specimen collections, in certain cases, may be a combination of different kinds.

1. International herbaria keep specimens from all over the world. Many of the world's largest herbaria are of this type (Table 3).
2. National herbaria keep specimens from all areas within a specific country. Many national herbaria may also hold specimens from neighbouring countries that share the same flora (Table 4).
3. Regional herbaria keep specimens from a specified region or province within a country.
4. Local herbaria keep specimens from a small area within a region or province.
5. Teaching or research herbaria are attached to a university or academic institution and are generally a place where research staff and students deposit their collections or voucher specimens used in their research projects.
6. Private herbaria are kept by individuals or are attached to private lands, such as farms or privately-owned land.

There can be specialised collections within a particular herbarium, such as:

1. Very old historical collection by a person or group;
2. Voucher specimens for ecological, anatomical, cytological, chemical, or molecular studies; or
3. Special interest collection for groups of plants with a linking quality, for example, medicinal plants.

Herbaria of the World

According to the data in *Index Herbariorum* as of 1 December 2020 (Thiers 2020b), there are 3,426 active herbaria in 182 countries of the world, containing 396,204,891 specimens. Ironically, many of the largest herbaria are in the temperate, industrialised countries of Europe and North America (Table 3), holding most specimens (as well as associated taxonomic information) from the developing countries of the tropics, which contain most of the world's plant biodiversity.

More than 100 herbaria in the world have at least 400,000 specimens. The largest of all, the herbarium at Royal Botanic Gardens, Kew (K), was founded in 1852 and hosts up to 8,125,000 specimens (Thiers 2020a; Table 3). Many new herbaria are added each year but many others are also reported as discontinued (Thiers 2020a), often owing to a decrease or removal of funding. Nearly 50 per cent of the active herbaria are located in Europe and North America (including Mexico) and hold almost 70 per cent of all the world's herbarium specimens.

Southeast Asia has 75 herbaria with approximately 6.5 million specimens (Table 4). The largest herbarium in this part of the world is the Research Center for Biology, Bogor (BO) in Indonesia, established in 1841, with over 2 million specimens.

In the Philippines, there are at least 15 herbaria registered with the Index Herbariorum, totaling more than 465,000 specimens (Thiers 2020a). The Philippine National Herbarium (PNH) has the largest collection of over 200,000 specimens (Table 5). The original specimens in PNH from its founding in 1903 included important collections by E.D. Merrill, A.D.E. Elmer, the Bureau of Science (BS) series, and the Forestry Bureau (FB) series, many of which were type specimens. The bulk of these early collections, however, were burned during World War II and the herbarium was rebuilt in 1946.

Table 3. Largest herbaria in the world

Herbarium (Acronym)	Country	Number of Specimens	Year Founded
Royal Botanic Gardens, Kew (K)	United Kingdom	8,125,000	1852
Museum National d'Histoire Naturelle Paris (P)	France	8,000,000	1635
New York Botanical Gardens (NY)	USA	7,800,000	1891
Naturalis / Leiden (L), Wagenigen (WAG), Utrecht (U)	Netherlands	6,900,000	1852
Missouri Botanical Gardens (MO)	USA	6,600,000	1859
Conservatoire et Jardin Botaniques, Geneva (G)	Switzerland	6,000,000	1824
Komarov Botanical Institute, Leningrad (LE)	Russia	6,000,000	1823
Naturhistorisches Museum Wien (W)	Austria	5,500,000	1807
British Museum of Natural History (BM)	United Kingdom	5,200,000	1753

Herbarium (Acronym)	Country	Number of Specimens	Year Founded
Harvard University Herbaria (A, AMES, ECON, FH, GH, NEBC)	USA	5,005,000	1842
Smithsonian Institution (US, USNC)	USA	5,000,000	1848

Source: Thiers 2020a

Table 4. Largest herbaria in Southeast Asia

Herbarium (Acronym)	Country	Number of Specimens	Year Founded
Research Center for Biology, Bogor (BO)	Indonesia	2,000,000	1841
Singapore Botanic Gardens (SING)	Singapore	750,000	1880
National Center for Natural Sciences & Technology, Hanoi (HN)	Vietnam	700,000	1960
Forest Research Institute Malaysia, Kepong (KEP)	Malaysia	300,000	1908
Forest Herbarium, Bangkok (BKF)	Thailand	200,000	1905

Source: Thiers 2020a

Table 5. Largest herbaria in the Philippines

Herbarium (Acronym)	Country	Number of Specimens	Year Founded
Philippine National Herbarium (PNH)	Manila	200,000	1903
Jose Vera Santos Herbarium, Institute of Biology, U.P. Diliman (PUH)	Diliman	20,000	1908
Botanical Herbarium, Museum of Natural History, U.P. Los Baños (CAHUP)	Los Baños	66,000	1951
Forestry Herbarium, Museum of Natural History, U.P. Los Baños (LBC)	Los Baños	13,600	1910

Source: Yap et al. 2013, Thiers 2020a

Herbarium Building and Specimen Storage

The herbarium building or room is basically a storage area of plant specimens for scientific use. The type of storage and the overall conditions in which the specimens are kept will be significant in the resulting quality of the collections and the scientific work that can be done using them. In some cases, the herbarium is restricted to what space is practically available for use.

The following are important to consider when establishing an herbarium (Fosberg & Sachet 1965; Bridson & Forman 1989):

Location. Where possible, avoid areas susceptible to flooding, or near fire-prone structures or vegetation; aim for easy access for staff and visitors.

Construction. Preferably purpose-built; must be dry, safe, fire-proof, waterproof, earthquake-proof, and termite-proof. Must allow for expansion. Other activities related to the herbarium (receiving specimens, decontamination, drying, poisoning, heating, and deep freezing) should be in a separate building or room.

Essential facilities. Must provide for staff needs (rest area, food area, office space, and toilets). There should also be a reception area for visitors.

Workspace for staff and visitors. This includes but is not limited to a mounting room, separate rooms for spirit collections or carpological/seed/wood collections, a library of references, and computer terminals to access the herbarium database. Spirit collections in glass jars include flowers and soft fruits in preservative liquid. The carpological collection contains the parts of a plant that could not easily be pressed on a herbarium sheet: seeds, fruits as well as any plant parts which are too large to be incorporated into the main collection as a standard herbarium sheet.



A Spirit collection in the Forest Herbarium, Bangkok (2011); **B** Carpological collection in the Herbarium, Queen Sirikit Botanic Garden, Chiang Mai (2015)
Photos by Edwino S. Fernando

Internal design. The temperature should be ideal for staff efficiency, at around 22–24°C; humidity should be low, around 40–50 per cent, to reduce the risk of fungal infection of specimens and breeding of insects. Air-conditioners and dehumidifiers are recommended in the hot and humid tropics, such as in the Philippines. Lack of ventilation can be a health hazard - some areas in the herbarium can have high levels of vapor from insecticides or fungicides or alcohol from the spirit collections. Natural lighting is best and will be necessary to provide lighting in work areas for specimen examination. Electrical outlets should be readily accessible for stereomicroscopes, desk lamps, and laptop computers.

Fittings and fixtures. Floor surfaces should avoid carpets and linoleum where pests can shelter. Work surfaces should be close enough to the herbarium storage cabinets or boxes to minimise travel distance, often near windows (for natural lighting) and large enough to spread out specimens when sorting. Cabinets or boxes should have shelves that are 2 cm deeper or wider than the herbarium sheets; some specimens are placed in fabricated carton boxes, then filed and arranged in open metal shelves (Figure 5); compactors (as mobile storage units) can help maximise use of space.



Figure 5.
Herbarium specimens
placed in carton boxes and
filed on open metal shelves,
Herbarium, Singapore
Botanic Gardens.
Photo by Edwino S. Fernando

Fire precaution. Herbarium specimens are highly valuable scientific material. Strict fire precautions must be enforced against smoking or use of naked flames. Regularly maintained fire extinguishers must be placed in all areas of the herbarium.

Pests and treatments. Herbarium specimens must be protected from damage by insect pests. Threats from insect pests in herbarium specimens are highest in the tropics. High temperature and humidity levels allow pests to multiply. Develop a programme to prevent infestation by

destructive insects with due consideration of potential hazards to the health of staff and visitors. Using poisonous substances is generally discouraged. Extreme caution should be taken when considering poisoning specimens as a semi-permanent deterrent to insect infestation.

Protection against fungal damage. The problem of fungal damage to herbarium specimens is particularly critical in very humid tropical areas causing decomposition of plant tissues and disfiguring morphological features. The use of dehumidifiers is helpful.

Processing and Accessioning Specimens

1. *The specimen.* By tradition, the herbarium specimen is a plant or plant part affixed to a 29 cm x 43 cm (11.5 x 16.5 inches) mounting sheet (Figure 6) (Fosberg & Sachet 1965; Bridson & Forman 1998). Recent developments often also include as 'herbarium specimens' the following: liquid-preserved material (spirit collection, usually 70% ethyl alcohol), wood samples, pollen, and spores on glass slides, and even photographs and line drawings associated with plant specimens deposited in the herbarium.

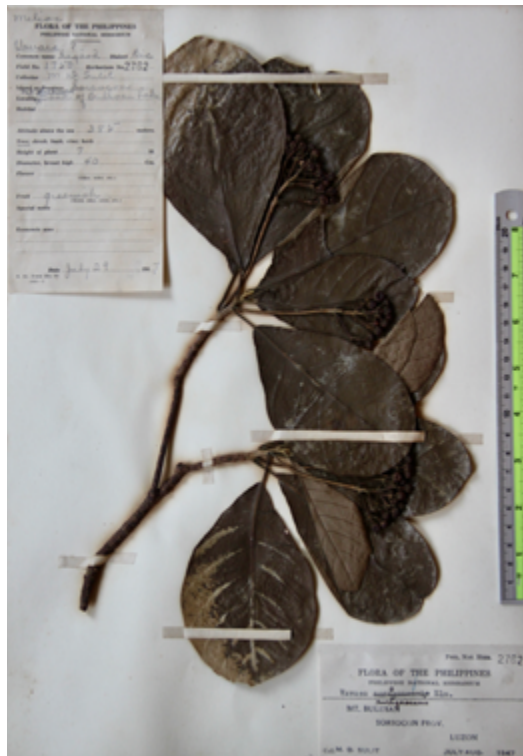


Figure 6.
Typical herbarium specimen.
Photo by Edwino S. Fernando

2. *Sources of herbarium specimens.* The entry of new specimens (usually unmounted) into an herbarium is normally from the following sources (Massey 1974):

Staff and student collections. Collections may be for floristic, monographic, or other botanical studies. Staff and students at institutions, which have no herbarium or only teaching or reference collections, may choose to document their studies by sending specimens to other institutions. These specimens may be sent either as gifts or loans and published papers should always include the name of the herbarium where vouchers are deposited.

Exchange. Duplicates or special collections are exchanged among various herbaria, usually on a one-for-one basis. This is one of the major means of adding to a collection and an efficient way of getting needed materials at minimal costs to all concerned.

Gifts or donations. Some treat receipt of all specimens other than exchange or loans as gifts or donations while others include only those for which no staff services are required. These may range from an entire herbarium to a few specimens sent to a staff specialist.

Loans. Loans are generally either temporary (short term, e.g., study of specific taxa for preparation of a revision or monograph) or indefinite (permanent, e.g., the loan of an entire herbarium from one institution to another). The latter is a particularly desirable procedure for historic and other collections, which may be for the most part inaccessible to most botanists or where proper housing facilities are lacking. These can often be loaned to another institution and still have the original name maintained.

Purchases. An uncommon event since usually private or institutional herbaria are donated or are deposited on permanent loan at an institution. Most purchases today involve collections from specific areas (particularly the tropics). In some cases, an institution may help to sponsor a collecting trip or provide free journal subscriptions to the herbarium in exchange for a set of specimens.

Identification service. Some herbaria may be willing to provide this service and send specimens for identification only with prior arrangement. In general, the specimens sent for identification are kept by the receiving herbarium, unless other arrangements are made (e.g. return unwanted specimens). Specimens kept should be of such quality that they are desirable as specimens and should not be sent without some prior agreement. Requests for identification services should be reasonable in terms of the time required and number of specimens to be identified.

Use of Herbarium, Operation, and Maintenance

Herbaria are for use regardless of type of collection — scientific research, identification, or teaching. When locating material within a collection (information retrieval), every effort should be made towards maximum efficiency without endangering the collection.

The following are useful to consider (after Fosberg & Sachet 1965; Bridson & Forman 1989):

Visitors. People wishing to use the herbarium should provide prior notice in advance of their expected arrival. Their email should indicate their full name and address of affiliated institution, contact details, and the plant families or genera they wish to examine.

Herbarium guide. The printed guide to the collection explains the systematic and geographic arrangement employed by the herbarium (often also prominently displayed near the herbarium cabinets). It should also include the colour code of folders, the list of families with numbers and case number, the map of case arrangement, the location and arrangement of special collections, and a generic catalogue. The guide should also contain herbarium policy

statements concerning re-shelving of materials, handling of specimens (see further below), use of equipment and other facilities, loan procedures, and other pertinent information.

Specimen use. Always treat the specimens as a priceless scientific and historic resource. Do not remove anything from the specimens unless you have expressed permission to do so. Do not write anything on the genus covers, herbarium sheets, or specimen labels. Do not treat a genus cover as a book or magazine, flipping the specimens over so that they are lying face downwards. Specimens should be face-up at all times. Do not leave unstable stacks of specimens on the tables, and always cover specimens when not working with them.

Handling herbarium specimens. Always handle specimens with care. Hold by both sides. Keep sheets flat. Never shuffle specimens – underlying specimens can be cut by the edges of those on top. Never align the specimens in a cover by holding it upright and striking one edge of the cover on the table. Do not turn specimens like the pages of a book or magazine. Check that all sheets are aligned before returning to their cabinets. Do not bend specimens to examine them under a stage microscope. Use a long-armed microscope or hand lens. Never rest books, heavy objects, or your arms or elbows on unprotected specimens. Place loose specimen fragments in a paper packet and fasten it to a sheet. Replace genus and species covers in the correct sequence. Pull forward the genus cover immediately below the covers being removed so that they are easy to replace. Do not force too many specimens into a shelf or box. Always shut herbarium cabinet doors firmly. Put specimens away as soon as possible. If specimens are to be left out, always leave them covered.

Loan of specimens. Most herbaria would lend materials only to other herbaria, not to individuals. Make sure that loan agreements exist before requesting a loan. For the most part loans should be requested after some preliminary work on the taxa concerned has been done so that unduly long-term loans are avoided (loan periods are generally for six months). Requests should be for reasonable amounts of material and for specific taxa rather than communities or floristic provinces. Loans should be requested by the curator or director of the herbarium for study by a specific person and should be addressed to the director or curator of the lending institution. Special permission should be obtained if fragments, pollen, leaf for DNA extraction, or other materials are to be removed.

Specimen care. Where possible, backlogs, which in turn create storage and infestation problems and consequently endanger specimens, are to be avoided. Inaccessible specimens are of no value. Specimens in need of repair and remounting should receive prompt attention as soon as they are noted. Some institutions repair all material before it is loaned or before re-shelving after it has been used. Repairs should be made as they are noted by persons filing new acquisitions and inserting new genus covers. Each year, the herbarium may need to be closed for several weeks for general repairs and to treat fungal damage and insect infestations on specimens.

Sorting and filing of specimens. Specimens are sorted to family, genus, and species. Additional sorting may be necessary if geographic regions are recognised in the collection. Specimens are then filed in heavy bristol folders called 'genus covers' (24 x 16.5 inches; 61 x 42 cm).

Arrangement of specimens. The specimens are arranged in the cabinets following a system, either (a) alphabetical by families: the advantage of an alphabetical system is that it is easy for the non-specialist to find taxa; disadvantage is that related, and therefore similar taxa are placed far apart, so that identification by matching becomes more difficult, or a (b) recent phylogenetic system (e.g. APG 2016) where phylogenetically related families and genera are placed close to one another, facilitating identification by matching. This may be difficult for the non-specialist to find families and to incorporate new material into the collection. A major change in the phylogenetic arrangement, resulting from recently published research, can make it necessary to change the physical arrangement of much or all of the collection. Because of their great importance and to protect them from regular handling, type specimens are placed in special red-coded folders and usually kept separate from the rest of the specimens in the herbarium.

Digitisation of herbarium specimens. Because herbarium specimens are key resources for documenting plant biodiversity their digitisation has become an important component of herbarium management. Digitisation includes both making digital images of the specimen and databasing the data associated with the specimen. Such activity will make the data more broadly available to the scientific community and general public accessible through the internet (Harris & Marsico 2017). Some digitisation workflows and specimen imaging methods have been suggested (Tulig et al. 2012; Takano et al. 2019). The entire digitisation process may take a significant time to complete and thus may be grouped into a several steps: predigitisation curation, imaging the specimens, databasing label and identifying information, and georeferencing locality information (Barkworth & Murrell 2012; Nelson et al. 2015). Any kind or quantity of data from a collection of herbarium specimens in the form of label data or images is useful and may be made available online at any stage of the digitisation process.

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Glossary

- absorbance** – amount of light absorbed by a sample. The quantity is measured by spectrophotometers to determine the purity and concentration of a certain molecule.
- agarose** – heteropolysaccharide extracted from a red seaweed. Adding water and subsequently boiling it will produce a gel used for agarose gel electrophoresis.
- bioinformatics** – branch of science that deals with storing, annotating, and analysing biological data using computer software programmes.
- cell lysis** – disruption of cell membranes using detergents and enzymes.
- chromatogram** – visual representation of a DNA sequence produced by a sequencing machine, with nucleotide bases expressed as coloured peaks.
- ClustalW multiple alignment** – computer algorithm that aligns DNA sequences.
- consensus sequence** – DNA sequence resulting from the assembly of the forward and reverse sequences.
- DNA barcode marker** – DNA sequences with a known physical location on a genome (e.g. *rbcL*, *matK*).
- DNA barcoding** – using specific DNA segments in the genome to rapidly and accurately identify an unknown individual to known species.
- DNA barcoding gap** – when the maximum intraspecific difference is smaller than the minimum interspecific difference.
- DNA Ladder** – used in gel electrophoresis as a reference for the size of the PCR products.
- DNA Primers** – short oligonucleotide sequence that binds to a DNA strand and serves as a starting point for DNA replication.
- elution** – process where an aqueous buffer is applied to the silica membrane of spin columns to release the pure DNA.
- flow-through** – liquid that passed through the silica membrane in spin columns.
- gel electrophoresis** – technique used to separate DNA strands of varying lengths by applying an electric field to DNA samples loaded in a gel.
- genomic DNA** – total genetic information of an organism.
- genus cover** – acid-free, lignin-free, light tan board that resembles a folder that is used to organise species of the same genus.
- herbarium press** – portable equipment used to flatten plant samples.
- herbarium voucher** – collected, pressed, and preserved plant sample. A voucher specimen may later be examined to verify the identity and origin of the plant sample.
- homology** – similarity of DNA sequences due to shared ancestry.
- lysate** – products of cell lysis.
- Materials Safety Data Sheet** – lists the potential hazards of a specific chemical or product, and information about the storage, handling, and emergency procedures to be taken in case of accidents related to the handling of the chemical or product.
- negative control** – PCR mix containing no DNA template and used to confirm absence of DNA contamination.
- phylogenetic tree** – diagram or graphical representation of evolutionary relationships among organisms.
- polymerase chain reaction (PCR)** – laboratory technique that produces many copies (usually millions) of DNA from a few copies of starting DNA template.
- sequence alignment** – sequence processing step that matches the homologous positions of DNA sequences to allow comparisons between multiple sequences.
- sequence assembly** – sequence processing step that compares the forward and reverse sequence side by side to come up with a consensus sequence.
- threshold value** – established value wherein if the genetic difference between an unknown individual and a known species in the database is lower than this value, then these two taxonomic units are interpreted to belong to the same species.
- trace files** – output files containing the raw data coming from sequencing machines. The raw data usually contain the sample name, chromatograms, and DNA sequences.

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